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1995

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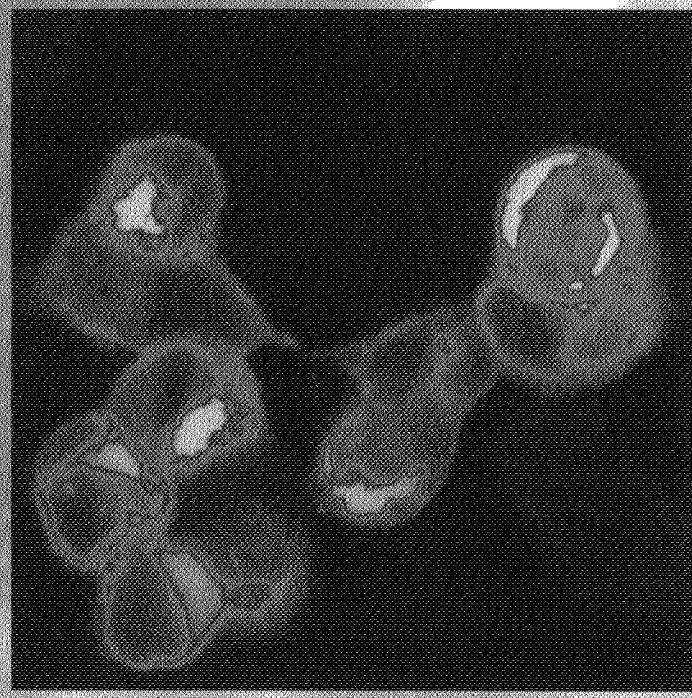

Martine Smit

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Cover design: Jos van den Broek (Micromys, Leiden)
Cover: Visualization of epitope-tagged histamine H_2 receptors in permeabilized human embryonal kidney cells (Chapter 8)

The studies presented in this thesis were performed in the Department of Pharmacochemistry, Division of Medicinal Chemistry, Vrije Universiteit, Amsterdam, The Netherlands. The Department of Pharmacochemistry is associated with the Leiden/Amsterdam Center for Drug Research (LACDR).

VRIJE UNIVERSITEIT

DYNAMIC REGULATION OF THE HISTAMINE H_1 AND H_2 RECEPTORS

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
prof.dr E. Boeker,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der scheikunde
op maandag 16 oktober 1995 te 15.45 uur
in het hoofdgebouw van de universiteit,
De Boelelaan 1105

door

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geboren te Decatur, Georgia
Verenigde Staten

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Copromotor:	dr R. Leurs
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Contents

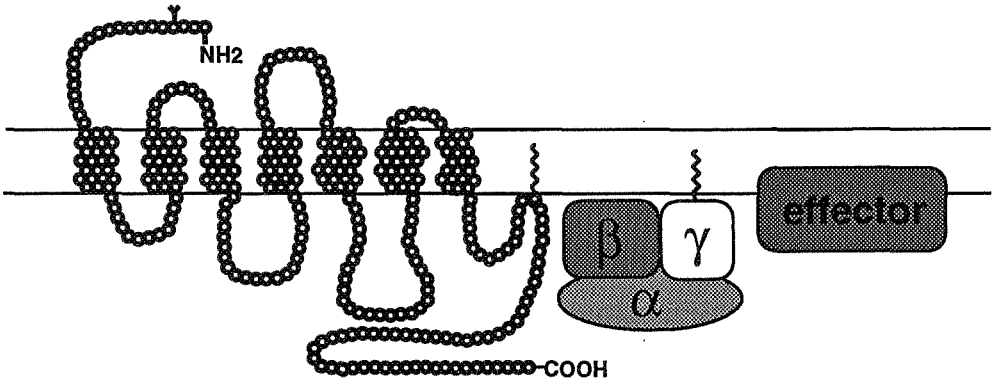

Fig.1. Schematic representation of a GPCR, a heterotrimeric G protein and effector enzym.

since deletion or modification of this region results in a receptor that still binds ligands but can not generate a response. Besides the third cytoplasmatic loop, other cytoplasmatic domains (e.g. second intracellular loop and cytoplasmatic tail) were also found to contribute to receptor G-protein interaction (Gudermann *et al.*, 1995, Moro *et al.*, 1993, Zhu *et al.*, 1994).

Upon agonist binding, the GPCR is thought to form a high affinity complex with the agonist and the heterotrimeric G-protein, consisting of an α, β and γ subunit (ternary complex model, Fig. 1) (De Lean, 1980). This receptor G-protein interaction causes the release of GDP from the guanine nucleotide binding site on the G_{α}-subunit, allowing GTP to bind. The GTP-bound form of the G-protein (α subunit) dissociates from the receptor and activates a specific effector protein, which in turn is responsible for the modulation of cellular activity. In recent years it has become evident that different G-proteins (Birnbaumer, 1993, Spiegel, 1992) exist, whereas also βγ subunits were found to be responsible for the activation of various cellular processes (Clapham and Neer, 1993, see also section 1.3.1). Moreover, also effector enzymes such as adenylyl cyclase and phospholipase C consist of a large family of isoenzymes (Cockcroft and Thomas, 1992, Iyengar, 1993, Tang and Gilman, 1992). The interaction of the GPCRs with the intracellular biochemical pathways is clearly very complex (Milligan, 1993). As such, the class of GPCRs can drastically influence the cellular activity. It is therefore important for the responsiveness of the biological system to be tightly controlled. GPCRs have long been known to be under control of negative feedback mechanisms (Collins, 1993, Lohse, 1993). These negative feedback mechanisms cause a loss of cellular responsiveness despite activation of the GPCR, a process referred to as desensitization. This phenomenon may also become apparent under pathophysiological conditions and after drug therapy (see section 1.3). As such it may have consequences for the development of effective drug therapy.

1.1.2 Histamine receptors

One of the many neurotransmitters acting via GPCRs is the monoamine histamine. In the CNS this amine is synthesized in a restricted population of neurons located in the tuberomammillary nucleus of the posterior hypothalamus (Airksinen *et al.*, 1992). These neurons project diffusely to most cerebral areas and have been implicated in several functions of the brain of mammalian species (e.g. sleep/wakefullness, hormonal secretion, cardiovascular control, thermoregulation) (Schwartz *et al.*, 1991). In various peripheral tissues histamine is stored in mast cells, basophils, enterochromaffin cells and probably also in specific neurons. In e.g. the gastric mucosa histamine is, depending on the species, stored in enterochromaffin-like cells or specific mast cells, which in response to gastrin and acetylcholine release the amine (Rangachari *et al.*, 1992). Histamine in turn stimulates the gastric acid secretion by parietal cells through interaction with specific receptors (Bertaccini and Coruzzi, 1992).

Histamine stored in mast cells also plays an important role in the pathogenesis of various allergic conditions. After mast cell degranulation, release of histamine leads to various of the well-known symptoms of allergic conditions in the skin and the airway system (Barnes, 1991, Ring *et al.*, 1985). Based on these observations histamine is considered as one of the important mediators of allergy and inflammation.

Initially, research in the histamine field was completely focussed on the role of histamine in allergic diseases indeed and this intensive research resulted in the development of several potent "anti-histamines" (e.g. mepyramine), which were useful in inhibiting certain symptoms of allergic conditions (see for references Janssens, 1993). The observation that these "anti-histamines" could not antagonize histamine-induced effects at the stomach and the heart, led Ash and Schild in 1966 to the hypothesis that histamine should act via at least two distinct receptor subtypes, referred to as the histamine H_1 and H_2 receptor (Table 1) (Ash and Schild, 1966). This hypothesis became generally accepted when Black *et al.* (1972) succeeded in the synthesis of a series of new compounds (e.g. burimamide), which were able to block the effects of histamine on the stomach and the heart. These H_2 receptor antagonists proved to be very useful in the therapy of gastric ulcers (Bertaccini and Corruzzi, 1992, Deakin and Williams, 1992). In recent years it became apparent that histamine is not only a mediator of several pathophysiological conditions, but also functions as a neurotransmitter (see reviews Hill, 1990, Leurs *et al.*, 1995a, Schwartz *et al.*, 1991). As found for many other neurtransmitter systems, a presynaptic receptor (H_3) for histamine appeared to exist (Table 1) (Arrang *et al.*, 1983). The identification of this new histaminergic receptor subtype in 1983 by Arrang and colleagues gave rise to a new field of interest for both pharmacologists and medicinal chemists. Although the development of the molecular biology of GPCRs has led to a dramatic increase of receptor subtypes for most of the known neurotransmitters (Savarese and Fraser, 1992), the subclassification of the histamine receptors is still based on the traditional pharmacological methods and has not been questioned yet by molecular biological approaches.

Table 1 Pharmacological definition of the three histamine receptors

	H_1	H_2	H_3
localization (function)	• smooth muscles in airways, intestine, blood vessels (contraction) • CNS (sleep/wakefulness, food intake, thermoregulation) • heart (pos. inotropic, pos. chronotropic effect)	• heart (pos. inotropic, pos. chronotropic effect) • stomach (gastric acid secretion) • uterus (contraction) • CNS (neuroendocrinological processes)	• CNS (inhibition histamine synthesis, inhibition neurotransmitter release) • lung (inhibition histamine synthesis, inhibition neurogenic contraction) • intestine (inhibition neurotransmitter release) • stomach (inhibition gastric acid secretion)
Effectors	IP_3/DAG	cAMP (increase)	?
agonists	2-thiazolylethylamine 2-phenylhistamine	dimaprit amthamine	(R)-α-methylhistamine imetit immepip
antagonists	mepyramine triprolidine	ranitidine tiotidine	thioperamide clobenpropit
radioligands	[^{3}H]mepyramine	[^{125}I]iodoaminopotentidine [^{3}H]tiotidine	[^{125}I]iodophenpropit [^{125}I]iodoproxyfan

Histamine H_1 receptors are found in smooth muscles of airways, intestine and blood vessels, where activation leads to contraction. They are also present in the central nervous system (CNS), where they are responsible for e.g. sleep/wakefulness, food intake, thermoregulation, and in the heart where they induce positive inotropic and chronotropic effects (Hill, 1990, Leurs *et al.*, 1995a, Schwartz *et al.*, 1991). The biological actions of H_1 receptors are mediated by stimulation of the phosphoinositide pathway (see section 1.2.2). Histamine H_2 receptors are mostly located in heart, stomach and CNS (McNeill, 1984, Schwartz *et al.*, 1991, Traiffort *et al.*, 1992a). Smooth muscles of airways and blood vessels also contain H_2 receptors (Barnes, 1991, Toda, 1987). The effects, including positive chronotropic and inotropic effects on the heart, activation of gastric acid secretion, inhibition of contraction of the uturus, relaxation of smooth muscle preparations and involvement in neuroendocrinological processes in the brain are thought to be mediated by activation of the adenylyl cyclase system (Hill, 1990, Leurs *et al.*, 1995a, Schwartz *et al.*, 1991) (see section 1.2.2). Histamine H_3 receptors are primarely located on neurons in the CNS, intestine, lung and stomach. In the brain H_3 receptor stimulation leads to the inhibition of histamine synthesis and also inhibits the release of several neurotransmitters (histamine, dopamine, serotonin, noradrenaline, acetylcholine) (Hill, 1990, Leurs *et al.*, 1995a, Schwartz *et al.*, 1993). At present the signal transduction pathway for the H_3 receptor-mediated actions is not known, although radioligand binding studies suggest a role for a G-protein in the H_3 receptor activation mechanism (Jansen *et al.*, 1994). Moreover, an inhibition of the phosphoinositide metabolism has been reported in HGT-1 gastric carcinoma cells (Cherifi *et al.*, 1992).

Pharmacological studies (radioligand binding studies, biochemical measurements) have indicated that the histamine H_1 and H_2 receptor, and most likely also the H_3 receptor, belong to the family of GPCRs. Evidence for these findings was provided by the cloning of the genes encoding the H_1 and H_2 receptor from different species (see section 1.2.1 and 1.2.1). The predicted amino acid sequences revealed that both receptor proteins belong to the GPCR family. The availibility of the genes encoding the histamine H_1 and H_2 receptor provide the possibility to obtain stable cell lines expressing high densities of the cloned histamine receptors (Fukushima *et al.*, 1993, Leurs *et al.*, 1994a,c, Iredale *et al.*, 1993, Traiffort *et al.*, 1992b). Detailed pharmacological characterization of the histamine H_1 and H_2 receptor in tissues or cell lines expressing endogenous histamine receptors was often hampered by the low expression of receptor proteins and lack of homogeneity in case of tissue preparations. The use of transfected cell lines and mutant receptors now provide the opportunity to examine structural requirements and mechanisms underlying regulation of histamine receptor function. By means of site-directed mutagenesis studies amino acids have been identified involved in the binding of histaminergic ligands to the H_1 and H_2 receptor (Gantz *et al.*, 1992, Leurs *et al.*, 1994b, Ohta *et al.*, 1994). Future studies will further define the ligand-binding domain and determine the receptor regions involved in G-protein coupling.

In the following section attention is paid to the molecular properties of both the histamine H_1 and H_2 receptor (section 1.2.1), describing the biochemical aspects of the receptor proteins and recent findings revealed by molecular biological techniques. The signal transduction pathways activated by both receptors are outlined in section 1.2.2. The phenomena of receptor regulation of the family of GPCRs are described in section 1.3.1 which is followed by a description of the regulation of the histamine H_1 and H_2 receptors (section 1.3.2). The clinical aspects of desensitization and implications for drug therapy will be discussed in section 1.3.3. The aim of this thesis will be outlined in the final section 1.4.

1.2 Molecular aspects of the histamine H_1 and H_2 receptor

1.2.1 Molecular properties of the histamine H_1 and H_2 receptor

Over the past few years, remarkable advances have been made in our understanding of the molecular properties of the histamine H_1 and H_2 receptor. First, the availibity of H_1 and H_2 receptor photoaffinity labels resulted in an improvement of our knowledge concerning the biochemical properties of the respective receptor proteins (Ruat *et al.*, 1988, Ruat *et al.*, 1990b, Yamashita *et al.*, 1991b). Thereafter, molecular biological approaches have been implemented in histamine research. In the last four years the genes encoding for the H_1 (De Backer *et al.*, 1993, Fujimoto *et al.*, 1993, Fukui, *et al.*, 1994, Horio *et al.*, 1993, Moguilevsky *et al.*, 1994, Traiffort *et al.*, 1994, Yamashita *et al.*, 1991a) and H_2 receptor (Gantz *et al.*, 1991a,b, Ruat *et al.*, 1991) from several species have been cloned. The molecular biological approach has provided, and will do so in the future, the opportunity to examine many aspects of histamine receptor function, including structural features required for ligand binding, G-protein coupling and receptor regulation.

The H_1 receptor protein

Using [^{125}I]-iodoazidophenpyramine the H_1 receptor proteins in rat, guinea-pig and mouse brain were irreversibly labelled (Ruat and Schwartz, 1989, Ruat *et al.*, 1988, 1990a). Following SDS-PAGE analysis of the labelled proteins, two main polypeptides (56 and 41-47 kDa) were found to be specifically labelled (Ruat and Schwartz, 1989). Based on experiments with protease inhibitors, it was suggested that the H_1 receptor binding protein was represented by the 56 kDa peptide, whereas the other labelled peptide was probably a result of proteases action (Ruat and Schwartz, 1989). Using [^{3}H]-azidobenzamide, Yamashita *et al.* (1991b) recently found receptor peptides of similar size (53-58 kDa) to be labelled in bovine adrenal medulla membranes. Whereas the 56 kDa peptide was also found in guinea-pig lung and ileum, a peptide with a substantially higher molecular weight (68 kDa) was labelled in guinea-pig heart tissue (Ruat *et al.*, 1990a). Although at present no pharmacological differences have been

observed between the H_1 receptors from guinea-pig heart and brain tissue, these results suggest the occurrence of several isoforms for the H_1 receptor. Moreover, the molecular weight found for the H_1 receptor after photoaffinity labelling is in sharp contrast with the reported weight (38-40 kDa) of a purified [^{3}H]-mepyramine binding protein from DDT_1MF-2 smooth muscle cells (Mitsuhashi and Payan, 1989). Since several highly potent H_1 receptor antagonists possess only a moderate affinity for this binding protein, it is not yet clear whether the binding of [^{3}H]-mepyramine to these cells really represents H_1 receptor binding (Mitsuhashi and Payan, 1989, Dickenson and Hill, 1994).

Yamashita *et al.* (1991a) were the first to succeed in the cloning of the cDNA encoding for the bovine H_1 receptor. Using the approach of expression cloning (injection mRNA in Xenopus oocytes) a cDNA clone from a cDNA library of bovine adrenal medulla was isolated and expressed in both Xenopus oocytes and COS-7 cells (Yamashita *et al.*, 1991a). The expressed receptor protein specifically binds [^{3}H]-mepyramine and shows in displacement studies typical H_1 receptor characteristics (Yamashita *et al.*, 1991a). The cloned cDNA encodes for a 491 amino acid receptor protein (apparent molecular weight 56 kDa) with all of the structural features of a GPCR (seven transmembrane domains, N-terminal glycosylation sites, phosphorylation sites for protein kinase A and C, Fig. 2). Since it is known from biochemical studies that the H_1 receptor protein from guinea-pig brain is glycosylated (Garbarg *et al.*, 1985), the predicted molecular weight of 56 kDa is certainly underestimated.

Using the sequence information of the bovine H_1 receptor cDNA (Yamashita *et al.*, 1991) the genes/cDNAs for the rat (Fujimoto *et al.*, 1993), guinea-pig (Horio *et al.*, 1993, Traiffort *et al.*, 1994) and human (De Backer *et al.*, 1993, Fukui, *et al.*, 1994, Moguilevsky *et al.*, 1994) H_1 receptor have been cloned. These genes are all intronless and encode for proteins of respectively 486 (rat), 488 (guinea-pig) and 487 (human) amino acids. The homology between the several receptor proteins is quite high in the intracellular domains (~ 90 %), but differs importantly in the long third intracellular loop and also in the extracellular N-terminal tail. The various cloned genes should be regarded as real species homologues, although some small changes in pharmacology are noticed (De Backer *et al.*, 1993, Fujimoto *et al.*, 1993, Fukui, *et al.*, 1994, Horio *et al.*, 1993, Moguilevsky *et al.*, 1994, Traiffort *et al.*, 1994, Yamashita *et al.*, 1991a).

With the availability of the cloned genes new possibilities have emerged in the field of histamine research. Application of molecular biological techniques has allowed investigation of the structure-function relationships of the H_1 receptor protein (Ohta *et al.*, 1994, Leurs *et al.*, 1994b). On the basis of results of site-directed mutagenesis studies of various other aminergic receptors it is generally accepted that the binding of these small neurotransmitters mainly occurs in the transmembrane domains (Savarese and Fraser, 1992). With respect to the H_1 receptor recognition of histamine it was shown that an aspartate residue in the third transmembrane domain functions as a binding site for the protonated amino-function of histamine (Ohta *et*

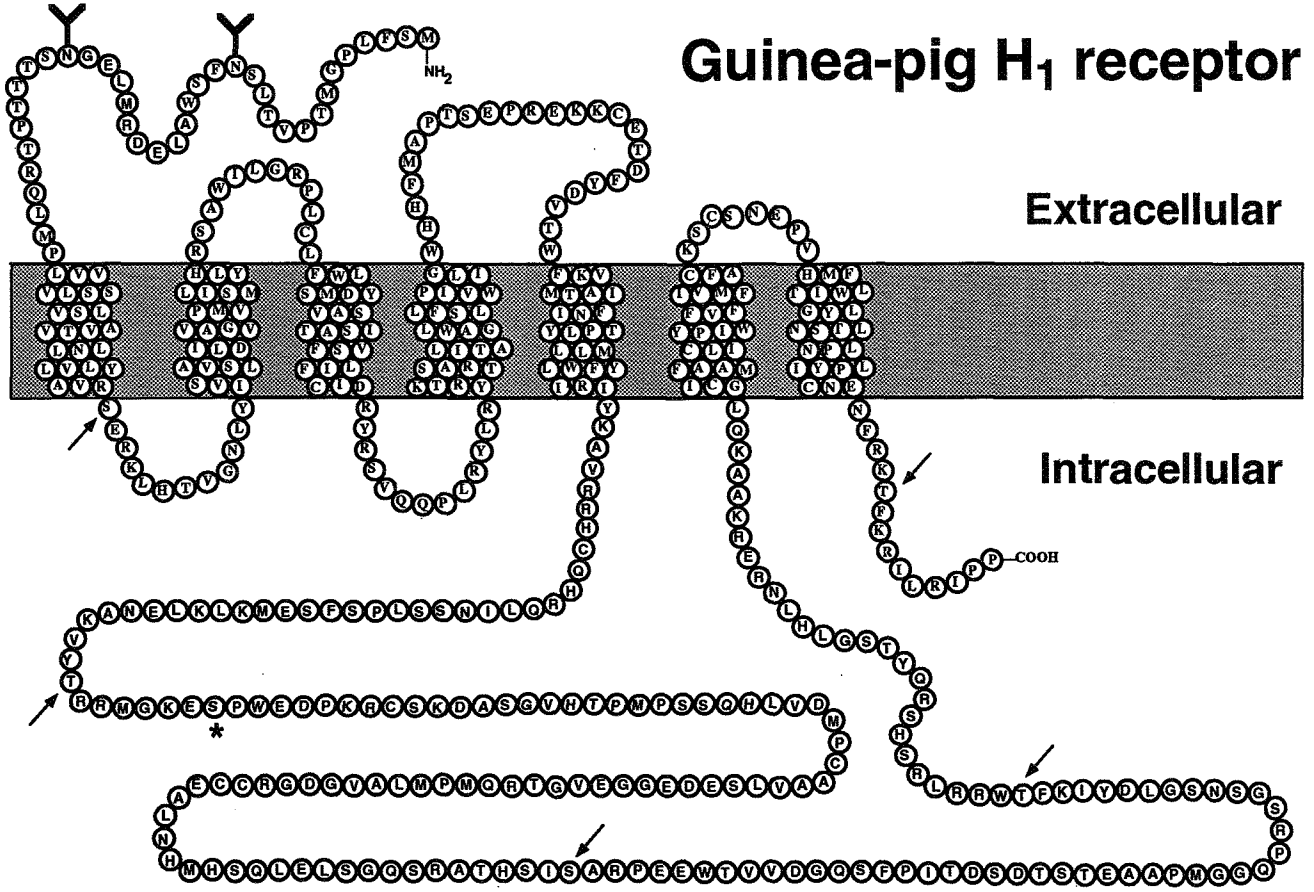

Fig. 2. Schematic representation of the guinea-pig histamine H_1 receptor. The amino acids are indicated by the single letter notation. A consensus sequence for asparagine-linked glycosylation is indicated. Moreover, possible PKA and PKC phosphorylation sites are indicated with an asterisk and arrows respectively.

al., 1994). Based on the findings on binding of catecholamines to the β_2-adrenergic receptor (Savares and Fraser, 1992), a threonine and asparagine in the fifth transmembrane domain have been suggested to be responsible for binding the imidazole moiety (Timmerman, 1992). Site-directed mutagenesis studies, substituting these amino acids to non-functional alanines showed that the threonine residue does not seem to be very important for the interaction with either agonist or antagonist (Leurs *et al.*, 1994b). In contrast, the asparagine residue appears to be very important for the interaction with histamine and 2-methylhistamine, a moderately selective H_1 receptor agonist, interacting most likely with the N^{τ}-nitrogen of the imidazole ring of histamine. Whereas on the basis of the agonist actions of various analogues, the N^{τ}-nitrogen of the imidazole ring of histamine has always been regarded as relatively non-functional, these ideas should now be reconsidered. In addition, the affinity of the selective agonist 2-(3-bromophenyl)histamine and the non-imidazole agonists 2-pyridylethylamine and 2-thiazolylamine for the $Asn^{207}Ala$ mutant was only slightly affected (Leurs *et al.*, 1994b), indicating that histamine and the non-imidazole agonists use different functionalities to bind to the receptor. More detailed studies should identify the amino acid residues of the H_1 receptor protein that are involved in the interaction with the aromatic ring of the 2-phenylhistamines and the N^{π}-nitrogen of the H_1 receptor agonists.

The H_2 receptor protein

Detailed biochemical information for the H_2 receptor has initially also been obtained by photoaffinity labelling studies. Using [^{125}I]-iodoazidopotentidine Ruat *et al.* (1990b) showed that the H_2 receptor binding peptide is probably a 59 kDa protein, although the purification of a [^{3}H]-tiotidine binding protein from human HGT-1 cells resulted in the identification of a 70 kDa protein (Reyl-Desmars *et al.*, 1991). Yet, the H_2 receptor nature of the [^{3}H]-tiotidine binding protein from these cells has not clearly been defined. Using an antipeptide antibody against the C-terminal sequence of the cloned canine H_2 receptor Fukushima *et al.* (1993) identified a band with an apparent molecular mass of 63 kDa to 95 kDa in the membranes from transfected Chinese Hamster Ovary (CHO) cells. The reason for this apparent heterogeneity is at present not clear, but could perhaps be explained by the use of different techniques to solubilize the receptor protein (M.J. Smit unpublished observations).

The cDNA sequence/genes encoding for the H_2 receptor has been elucidated for several species including man (Gantz *et al.*, 1991a,b, Ruat *et al.*, 1991, Traiffort *et al.*, 1995). Using the polymerase chain reaction with degenerate primers, based on the known sequence similarity of various GPCRs, and canine gastric parietal cDNA, Gantz *et al.* obtained the cDNA encoding for the canine H_2 receptor (Gantz *et al.*, 1991b). Soon thereafter, the intronless gene encoding for the rat (Ruat *et al.*, 1991) and the human H_2 receptor (Gantz *et al.*, 1991a) were rapidly cloned by means of homology screening. The cDNAs and various genes show considerable homology (80-90%) and are probably real species homologues

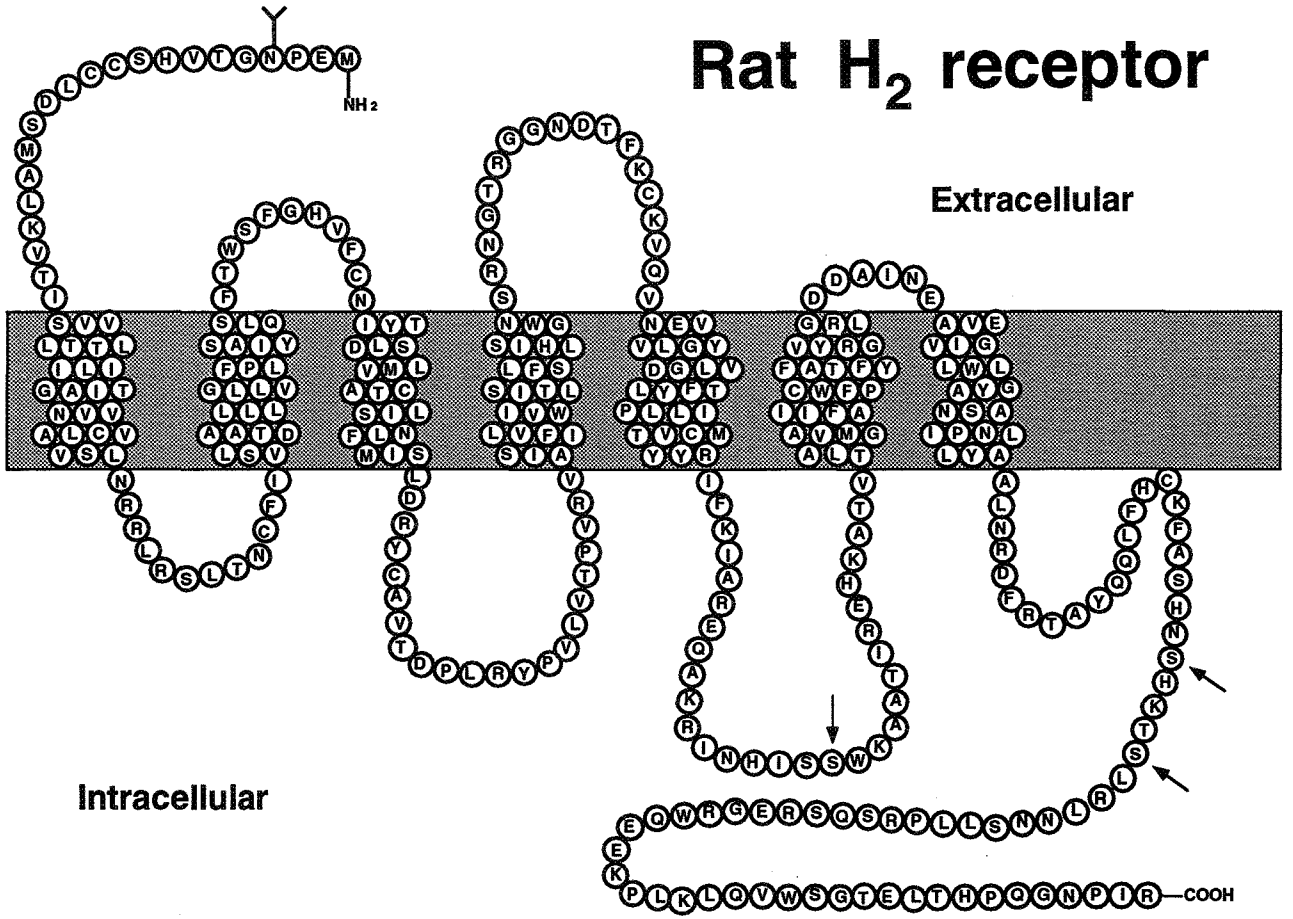

Fig. 3. Schematic representation of the rat histamine H_2 receptor. The amino acids are indicated by the single letter notation. A consensus sequence for asparagine-linked glycosylation is indicated. Moreover, possible PKC phosphorylation sites are indicated with arrows.

(Gantz *et al.*, 1991a,b, Ruat *et al.*, 1991, Traiffort *et al.*, 1995). The DNA sequences encode for a 359 (dog, man, guinea-pig) or 358 (rat) amino acid receptor protein, containing many of the structural features of GPCRs and apparent molecular weights of approximately 40 kDa. Since in the N-terminal extracellular tail a consensus sequence for N-linked glycosylation is present (Fig. 3), the actual molecular weight of the receptor will be significantly higher. Expression of the human and rat receptor gene in CHO cells was followed by an extensive pharmacological characterization (Leurs *et al.*, 1994a, Traiffort *et al.*, 1992b). Using the highly selective and potent radioligand [^{125}I]-iodoaminopotentidine Traiffort *et al.* (1992b) and Leurs *et al.* (1994b) revealed that respectively the rat and human H_2 receptor show a similar pharmacological profile as the H_2 receptor in guinea-pig heart, the commonly accepted H_2 receptor reference system, or in human brain. Although for the dog receptor protein extensive pharmacological data are not yet available it is very likely that the gene Gantz *et al.* (1991b) identified encodes for a classical H_2 receptor.

Site-directed mutagenesis of the canine H_2 receptor by Gantz *et al.* (1992) showed that an aspartate residue in the third transmembrane domain is also involved in the binding of the protonated amino function of histamine and H_2 receptor antagonists. Mutations of the aspartate and threonine residues located in the fifth transmembrane domain of the H_2 receptor protein to alanine residues resulted in a loss of [^{3}H]-tiotidine binding and a reduction of the histamine-induced cAMP production (Gantz *et al.*, 1992), indicating that the aspartate and threonine residues likely interact with histamine and the antagonist [^{3}H]-tiotidine. The interaction of the two residues with the antagonist is, based on studies with other biogenic amine receptors, however rather unexpected and certainly deserves future attention. Moreover, also the interaction of the two amino acids with histamine is not well defined; the mutated receptors require more detailed investigations with e.g. the recently developed non-imidazole agonists amthamine and amselamine (Eriks *et al.*, 1992, Van der Goot *et al.*, 1994).

1.2.2 Transmembrane signalling of the histamine H_1 and H_2 receptor

As already stated in section 1.1 efficient communication pathways exist between and within cellular entities. Histamine may be considered as a 'chemical messenger', responsible for the intercellular communication, whereas complex biochemical pathways are involved in the intracellular communication. GPCRs communicate with heterotrimeric G-proteins to direct the flow of information to effector molecules. The availability of the genes encoding for the histamine H_1 and H_2 receptor (see section 1.2.1) has allowed the expression of these receptor proteins into various cell lines. This approach permits detailed investigations of the several transmembrane signalling pathways that can be activated by the different receptors. Detailed insights of these signalling pathways are essential for a good understanding of the action of histamine in (patho)physiological processes.

Signal transduction of the H_1 receptor

The histamine H_1 receptor is, among other GPCRs, a Ca^{2+} mobilizing receptor. It is widely accepted that activation of Ca^{2+} mobilizing receptors is associated with the phospholipase C catalyzed hydrolysis of membrane inositide phospholipids (Michel, 1975). Receptor stimulation leads to the hydrolysis of phosphatidyl 4,5- biphosphate resulting in the formation of inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 was shown to mobilize Ca^{2+} from intracellular stores, whereas DAG was found to activate protein kinase C (PKC). For a detailed description of the various functions of these second messengers the interested reader is referred to a review of Berrigde (Berridge, 1992).

In accordance with the above, histamine induces production of inositol phosphates in several tissues, including brain, airway smooth muscle, intestinal smooth muscle, vascular smooth muscle and heart tissue (Barnes, 1991, Claro *et al.*, 1989, Donaldson and Hill, 1986, Orellano *et al.*, 1987, Sakuma *et al.*, 1988, Schwartz *et al.*, 1991). Moreover, guinea-pig brain regions showing the highest density of H_1 receptors displayed the largest phosphoinositide response (Carswell and Young, 1986, Daum *et al.*, 1983). However, in some tissues, guinea-pig ileum and neonatal brain, the H_1 response to histamine itself appeared to be masked by a H_1 antagonist insensitive component (Bailey *et al.*, 1987, Claro *et al.*, 1987, Donaldson and Hill, 1985).

In membrane preparations of both rat cerebral cortex and 1321N1 astrocytoma cells, H_1 receptor-stimulated phospholipid hydrolysis was found to be dependent on the presence of guanine nucleotides (Claro *et al.*, 1989, Orellano *et al.*, 1987). Studies in 1321N1 astrocytoma cells (Orellano *et al.*, 1987), HeLa cells (Tilly *et al.*, 1990a) and CHO cells stably expressing H_1 receptors (Leurs *et al.*, 1994c), showed the involvement of a pertussis toxin-insensitive G-protein. The actual nature of the pertussin toxin-insensitive G-protein remains unclear. However, the recent cloning of the H_1 receptor as well as identification of various G-proteins will allow a detailed investigation of this issue.

One of the physiological consequences of the production of inostol phosphates is the elevation of intracellular Ca^{2+}. The use of fluorescent Ca^{2+} indicators in various isolated cell systems has clearly demonstrated the increase of the intracellular Ca^{2+} concentration upon H_1 receptor activation (Bloemers *et al.*, 1993, Brown *et al.*, 1986, Jacob *et al.*, 1988, Johnson *et al.*, 1990, Kotlikoff *et al.*, 1987, Leurs *et al.*, 1994c, Matsumoto *et al.*, 1986, McDonough *et al.*, 1988, Oakes *et al.*, 1988, Rotrosen and Gallin, 1986, Smit *et al.*, 1992, Tilly *et al.*, 1990b). The histamine-induced Ca^{2+} response is characterized by a rapid transient rise of the intracellular Ca^{2+} concentration, which is followed by a sustained elevation of the Ca^{2+} concentration. Experiments in Ca^{2+}-free medium and with inorganic Ca^{2+} antagonists suggest that the sustained response is highly dependent on the influx of extracellular calcium, whereas the transient increase is caused by the release of Ca^{2+} from intracellular Ca^{2+} stores (Brown *et al.*, 1986, Jacob *et al.*, 1988, Johnson *et al.*, 1990, Kotlikoff *et al.*, 1987, Leurs

et al., 1994c, Matsumoto *et al.*, 1986, McDonough *et al.*, 1988, Oakes *et al.*, 1988, Rotrosen and Gallin, 1986, Tilly *et al.*, 1990b).

Since Ca^{2+} is involved in the regulation of many cellular functions, the increase of the intracellular Ca^{2+} concentration can explain a wide variety of pharmacological responses induced after stimulation of the H_1 receptor. First of all, elevation of intracellular Ca^{2+} levels lead to further stimulation of phospholipase C, most likely the phospholipase Cβ subtype as it is known to be sensitive to Ca^{2+} (Cockcroft and Thomas, 1992). Moreover, the histamine-induced production of inositol phosphates in both brain and tracheal slices (Alexander *et al.*, 1990, Baird *et al.*, 1989) and CHO cells stably expressing the guinea-pig H_1 receptor (Leurs *et al.*, 1994c) was shown to be highly dependent on the influx of extracellular Ca^{2+}.

Besides the activation of phospholipase C, histamine-induced increase of Ca^{2+} seems to induce the production of nitric oxide. In various vascular preparations, endothelium-dependent relaxation is observed upon H_1 receptor activation, which appeared to be related to the production of nitric oxide (Satoh and Inui, 1984, Schoeffter and Godfraind, 1991, Toda, 1987, Van den Voorde and Leusen, 1984). In cultured bovine aortic cells the actual generation of nitric oxide could be measured after stimulation of histamine (Schmidt *et al.*, 1990). In a variety of airway and heart preparations H_1 receptor activation was shown to induce the production of cGMP, which may be ascribed to generation of nitric oxide (Casale *et al.*, 1985, Duncan *et al.*, 1980, Hattori *et al.*, 1988, Leurs *et al.*, 1991a, Sertl *et al.*, 1987, Yuan *et al.*, 1993). Thus, some vascular responses to histamine may be explained by the generation of nitric oxide, which appears to be highly dependent on the mobilization of intracellular Ca^{2+}.

Another consequence of the histamine-induced mobilization of Ca^{2+} is the generation of arachidonic acid metabolites, prostacyclin and thromboxane A_2, due to liberation of arachidonic acid from the phospholipids (Leurs *et al.*, 1994c, Lewis Baenziger *et al.*, 1980, Resink *et al.*, 1987). In CHO cells transfected with the guinea-pig H_1 receptor and HeLa cells the release of arachidonic acid appeared to be phospholipase A_2 dependent (Leurs *et al.*, 1994c). In these CHO cells the histamine-induced activation of phospholipase A_2 was found to be partially sensitive to pertussis toxin (Leurs *et al.*, 1994c). In contrast, in these cells the regulation of phospholipase C was completely insensitive to pertussis toxin (Leurs *et al.*, 1994c). These findings are in agreement with the observations described by Murayama *et al.* (1990), who reported that in human and rabbit platelets histamine stimulates the release of arachidonic acid via a pertussis toxin sensitive pathway without the generation of inositol phosphates. In HeLa cells, on the other hand, the phospholipase A_2 dependent release of arachidonic acid appeared to be insensitive to pertussis toxin (Leurs *et al.*, 1994c). Thus, the H_1 receptor-mediated activation of phospholipase A_2 in transfected CHO cells may be ascribed to two different pathways; arachidonic acid can be stimulated via an elevation of the intracellular Ca^{2+} concentration (as in HeLa cells) and via interaction of phopholipase A_2 with a pertussis toxin sensitive G-protein (as in platelets).

Last, the increase of intracellular Ca^{2+} is most likely involved in the regulation of cAMP levels. H_1 receptor stimulation seems to modulate cAMP responses via histamine H_2, adenosine A_2 and VIP receptors (Al-Gadi and Hill, 1987, Donaldson *et al.*, 1989, Garbarg and Schwartz, 1988, Magistrati and Schorderet, 1985, Marley *et al.*, 1991). The exact mechanism involved in the elevation cAMP remains to determined, however, a role for both Ca^{2+} and PKC has been suggested (Hill and Donaldson, 1992). Yet, recent findings in CHO cells expressing the guinea-pig H_1 receptor showed that PKC nor Ca^{2+} did account for the observed elevation of cAMP levels (Leurs *et al.*, 1994c). The latter observations could possibly be explained by stimulation of adenylyl cyclase by G-protein βγ-subunits (Iyengar, 1993).

In conclusion, the histamine H_1 receptor is coupled to the phospholipase C-dependent inositol phosphate pathway, thereby inducing a rise in the intracellular Ca^{2+} concentration. The latter can explain the histamine-induced production of other second messengers such as cGMP, cAMP and arachidonic metabolites. Yet, it appears that responses such as modulation of cAMP and phospholipase A_2 activity can also be regulated by phospholipase C-independent pathways. The way, activation of the H_1 receptor leads to these responses, requires further detailed mechanistic investigations.

Signal transduction of the H_2 receptor

It is generally accepted that the histamine H_2 receptor is coupled to the adenylate cyclase system. A large number of reports showed that histamine increases the levels of cAMP in e.g brain, stomach and heart tissue of several species, including man (Agullo *et al.*, 1990, Bristow *et al.*, 1982, Foreman *et al.*, 1986, Hattori *et al.*, 1988, Hegstrand *et al.*, 1976, Johnson, 1992, Ozawa *et al.*, 1991, Ozawa and Segawa, 1988, Sarem-Aslani et *al.*, 1991, Schwartz *et al.*, 1991). Since the H_2 receptor mediates its response through activation of adenylate cyclase in membrane fractions in a guanyl nucleotide-sensitive manner (Johnson, 1992), an activation model similar to the well-studied β-adrenergic receptor system is often presented (Johnson, 1992). However, it should be emphasized that, although such a model is highly probable, no direct evidence is at present available for the interaction with a G_s-protein. In this respect it is interesting that Ozawa and co-workers suggested that a H_2 receptor-stimulated phospholipid methylation is responsible for a subsequent activation of adenylate cyclase (Ozawa and Segawa, 1988, Ozawa *et al.*, 1991). In both rat brain (Ozawa and Segawa, 1988, Ozawa *et al.*, 1987) and guinea-pig heart (Ozawa *et al.*, 1991) histamine was shown to rapidly stimulate the phospholipid methylation. The cAMP response in both tissues was significantly slower than the phospholipid methylation and completely dependent on the presence of the methyldonor S-adenosyl-L-methionine and reduced by an inhibitor of the phospholipid methyltransferases (Ozawa and Segawa, 1988, Ozawa *et al.*, 1991). Moreover, in rat brain the regional distribution of the histamine-induced phospholipid methylation closely paralleled the H_2 receptor-mediated cAMP response (Ozawa and Segawa, 1988). These data

are intriguing and suggest that the phospholipid response might be necessary for an effective coupling of the receptor to the G-protein responsible for the activation of the cyclase. Interesting in this respect is that also much earlier for the β-adrenergic receptor similar suggestions have been reported by Hirata and Axelrod in 1980. Yet, it should be noticed that currently, these observations are not drawing much attention, nor are within the field of the β-adrenergic receptor.

Although the linkage of the H_2 receptor to the adenylate cyclase is rather well accepted, some findings argue against a universal role of cAMP. In guinea-pig brain the regional distribution of H_2 receptor binding sites does not parallel the observed H_2 receptor-mediated cyclase acivation (Ruat *et al.*, 1990b). Moreover, Haas *et al.* (1978) observed a denervation hypersensitivity to histamine at the electrophysiological level, whereas under the same conditions the cAMP response was unaltered.

In view of these findings it is interesting that new signalling pathways have been described for the H_2 receptor. In differentiated HL-60 cells and HEPA cells, transfected with the canine H_2 receptor, a H_2 receptor mediated increase of the intracellular Ca^{2+} concentration was observed (Gespach *et al.*, 1982, Delvalle *et al.*, 1992, Mitsuhashi *et al.*, 1989). The increase of intracellular Ca^{2+} was found to result from the release of Ca^{2+} from intracellular stores (Delvalle *et al.*, 1992, Mitsuhashi *et al.*, 1989). The H_2 receptor-dependent Ca^{2+}-mobilization is probably due to the activation of phospholipase C, since histamine induced an increase of the levels of inositol 1,4,5-trisphosphate. In both cell lines these effects were found to be inhibited by cholera toxin but not by pertussis toxin, suggesting the involvement of a G-protein (Delvalle *et al.*, 1992, Mitsuhashi *et al.*, 1989). Recent reports in hamster DDT_1MF-2 cells and bovine trachea smooth muscle have demonstrated a cAMP-mediated inhibition of the production of inositol phosphates (Dickenson *et al.*, 1993, Hall and Hill, 1988, Hall *et al.*, 1989) The crosstalk between the cAMP cascade and phosphoinositide system could well explain the observed inhibitory effects induced by cholera toxin. Yet, in the HEPA cells, forskolin did not inhibit the histamine induced effects, suggesting the involvement of another mechanism (Delvalle *et al.*, 1992). The involvement of distinct G-protein can not be excluded.

Another cAMP-independent response was described for the cloned rat H_2 receptor expressed in CHO cells by (Traiffort *et al.* 1992b). Besides a massive production of cAMP upon H_2 receptor activation, an inhibition of the release of arachidonic acid induced by either constitutive purinergic receptors or a Ca^{2+}-ionophore was observed. The histamine-induced inhibition was potently inhibited by ranitidine (K_i = 0.16 μM) and also induced by dimaprit. Forskolin, prostaglandin E_1 or 8-bromo-cAMP did not mimick the effects induced by histamine indicating the involvement of a cAMP-independent process (Traiffort *et al.*, 1992b). Histamine did not significantly modify the features of the Ca^{2+}-responses to ATP. At present the actual mechanism for this response is unknown. Yet, one should be aware that this coupling to a new signalling system could be due to the high expression of receptors in these cells. In contrast, expression of the gene encoding the human H_2 receptor in CHO cells was

shown to be functionally coupled to adenylate cyclase, but did not influence the inositol phosphate turnover nor arachidonic acid release (Leurs *et al.;* 1994a). The expression level of the human H_2 receptor resembles a more physiological level of expression (Leurs *et al.*, 1994a, Chapter 7). Moreover, the observed sequence differences, in particular in the intracellular parts, between the rat and human receptor (Gantz *et al.*, 1991a, Ruat *et al.*, 1991b) might also be a possible explanation for the observed differences in signal transduction.

In conclusion, the H_2 receptor is coupled to the adenylyl cyclase-dependent production of cAMP. Yet, the observed breakdown of phosphoinosites, elevation of intracellular Ca^{2+} levels and modulation of phospholipase A_2 activity upon H_2 receptor activation, seem to be regulated via other cAMP-independent pathways.

1.3 Regulation of receptor function

1.3.1 Regulation of GPCRs

GPCRs are responsible for the generation of various second messengers that affect cellular functions and gene expression. It is therefore important for the responsiveness of the biological system to be tightly controlled. A major mechanism involved in the control of GPCRs is receptor desensitization (see reviews Collins *et al.* 1991, Collins, 1993, Lohse, 1993, Savarese and Fraser, 1992). Desensitization is a general biological phenomenon which is characterized by a rapid loss of responsiveness over time despite the continuous presence of a stimulus. At least three mechanistically and temporarily distinct processes are thought to contribute to the overall phenomenon of desensitization (Collins *et al.* 1991, Collins, 1993, Lohse, 1993). The first involves the functional uncoupling of receptors from their effector system shortly after agonist binding and is referred to as short-term desensitization. The second mechanism is the translocation of receptors from the cell surface into a membrane-associated compartment within a few minutes after agonist exposure. Such agonist-promoted endocytosis, also referred to as internalization or sequestration, has been proposed to play a primary role in the resensitization of receptors (Pippig *et al.*, 1995, Von Zastrow and Kobilka, 1992, Yu *et al.*, 1993). Finally, long-term exposure of a receptor to an agonist leads to receptor downregulation (also referred to as long-term desensitization), defined as the breakdown of receptor proteins.

In this context, it is also important to make a distinction between homologous and heterologous desensitization. When the loss of stimulatory activity in the pathway is specific for the receptor being activated, it is termed homologous desensitization. Specific modifications of the receptor protein itself are believed to be involved in the process of desensitization. In contrast, if the functions of other receptor systems are affected upon activation of one receptor, one refers to heterologous desensitization. Such a regulation can also be the result of specific

modifications of the receptor protein but usually is caused by alterations at levels distal to the receptor, e.g. G-protein or the effector enzyme.

The mechanisms underlying receptor desensitization have been studied most extensively for the β_2-adrenergic receptor (Collins, 1993). The understanding of this phenomenon has been facilitated by the early molecular cloning of the β_2-adrenergic receptor gene (Dixon *et al.*, 1986) and the utilization of site-directed mutagenesis. This receptor therefore often serves as a model system for the investigation underlying receptor desensitization.

Desensitization

One of the mechanisms underlying short-term receptor desensitization is thought to be associated with post-translational modification of the receptor protein by phosphorylation (Hausdorff *et al.*, 1990, Huganir and Greengard, 1990, Lefkowitz *et al.*, 1990). Specific receptor kinases seem to play crucial roles in the mechanism underlying homologous receptor desensitization, whereas kinases, such as protein kinase A (PKA) and protein kinase C (PKC) seem to be important in the heterologous type of desensitization (Huganir and Greengard, 1990, Lefkowitz *et al.*, 1990, Palczewski and Benovic, 1991).

For the β_2-adrenergic receptor homologous receptor desensitization was found to be associated with receptor phosphorylation catalyzed by a specific receptor kinase called β-adrenoceptor kinase (βARK), which specifically phosphorylates the agonist-occupied form of the receptor (Hausdorff *et al.*, 1990, Huganir and Greengard, 1990, Lefkowitz *et al.*, 1990). Besides βARK, a cofactor called β-arrestin was found to be involved in this process of desensitization (Lohse *et al.*, 1990, Pippig *et al.*, 1993). β-arrestin shares reasonable homology with the α-subunit of the G_s-protein. Moreover, β-arrestin shows only affinity for the phosphorylated β_2-receptor. Binding of β-arrestin to the phosphorylated β_2-adrenergic receptors inhibits the receptor-G_s interaction, thereby creating an uncoupled or desensitized state of the receptor.

Besides phosphorylating the β_2-adrenergic receptor, βARK was also found to phosphorylate other GPCRs, such as rhodopsin, the α_{2A} adrenergic receptor, the muscarine m_2, substance P receptor and platelet-activating factor receptor indicating that not only GPCRs which are positively coupled to adenylyl cyclase are substrate for this kinase (Benovic *et al.*, 1989, Lefkowitz *et al.*, 1990, Inglese *et al.*, 1993, Haga *et al.*, 1994, Kwatra *et al.*, 1993, Takano *et al.*, 1994). Application of molecular cloning techniques resulted in the isolation of related kinases (see for more details reviews Lefkowitz, 1993, Haga *et al.*, 1994). Therefore, a more general term was introduced: G-protein coupled receptor kinases (GRKs). To date, the family of GRKs is still expanding. Up to 6 cDNAs encoding GRKs have been isolated (see for references Lefkowitz, 1993, Haga *et al.*, 1994). The tremendous diversity of the GPCRs, compared to the limited number of GRKs, raises questions about the receptor specificity of the various GRKs. The GRKs showed differences in their ability to phosphorylate the β_2-adrenergic receptor or other GPCRs, suggesting that these kinases have distinct substrate

specificities. In addition, the level of expression and distribution of this class of GRKs differ. The expression of some GRKs is confined to restricted areas (e.g. GRK1 (or rhodopsin kinase) in retina and pineal gland, GRK4 (or IT11) in brain and testes), implying specific functions (see for references Lefkowitz, 1993, Haga *et al.*, 1994). Others, such as GRK2, are widely distrubuted throughout the mammalian central nervous system, suggesting broader roles in regulating a wide variety of neurotramsmitter receptors (Arriza *et al.*, 1992).

There is no strict consensus sequence for the phosphorylation by the GRKs, but it seems that acidic amino acids located 2 or 3 positions N-terminal to a serine or threonine are required for phosphorylation by GRKs (Onorato *et al.*, 1991). Removal of serine/threonine residues from the C-terminal tail of the β_2-adrenergic receptor by site directed mutagenesis resulted in decreased phosphorylation of the β_2-adrenergic receptor (Bouvier, 1988, Campbell, 1991). Moreover, removal of these residues by site-specific substitution or truncation of the C-terminal tail of the β_2-, α_{1B}-, substance P neurokinin NK_2, adrenergic and platelet-activating factor receptors were shown to attenuate the homologous desensitization (Alblas *et al.*, 1995, Lattion *et al.*, 1994, Lefkowitz, 1993, Sasakawa *et al.*, 1994, Takano *et al.*, 1994). For the α_{2A}-adrenergic receptor and m2 muscarinic receptor phosphorylation sites were shown to reside in the third intracellular loop (Liggett *et al.*, 1992, Dohlman *et al.*, 1987, Nakahata *et al.*, 1994). Recently, four consecutive serines in the third intracellular of the α_{2A}-adrenergic receptor were shown to be sites for GRK-mediated phosphorylation and to be critical for agonist-promoted desensitization (Eason *et al.*, 1995).

G-protein $\beta\gamma$ subunits ($\beta\gamma$) were shown to play a role in the translocation of some GRKs from cytosol to the membrane and the stimulation its enzyme activity, thus enhancing agonist-stimulated receptor phosphorylation and receptor desensitization (Inglese *et al.*, 1993, Haga *et al.*, 1994). The mechanism of activation appears to be largely due to the specific interaction of the isoprenylated $\beta\gamma$ complex (the γ subunit appears to be isoprenylated) with GRKs 2 and 3, which serves to target the kinases to the plasma membrane. These GRKs appear to possess $G_{\beta\gamma}$-binding domains, containing pleckstrin homology (PH) domains. PH domains, consisting of 100 amino acids forming a large binding pocket, have only recently been recognized as sites for interaction between proteins (Yoon *et al.*, 1994). If the various $\beta\gamma$ complexes show differential affinity for the various GRKs, this could be a mechanism for directing GRK isoforms to distinct GPCRs.

Unlike GRKs 2 and 3, GRK1 (or rhodopsin kinase) does not require $\beta\gamma$ subunits to translocate to the membrane, as in contrast to GRKs 2 and 3 GRK1 is isoprenylated at its C-terminus and does not require a secondary membrane anchor (Inglese *et al.*, 1993). GRKs 5 and 6 are also not activated by $\beta\gamma$ subunits suggesting a different mechanism for translocation (Benovic and Gomez, 1993, Kunapuli and Benovic, 1993). Kunapuli *et al.* (1994) have recently demonstrated that phospholipid-stimulated autophosphorylation may provide a new mechanism for activation of GRK5, as well as a potential mechanism for the *in vivo* targetting

to its substrates. Thereafter, DebBurman *et al.* (1995) reported that the GRKs 2 and 3 are also regulated by lipids via a mechanism that does not involve autophosphorylation. The effects of these lipids were prevented by the $G_{\beta\gamma}$-binding protein phosducin, which was found to inhibit phosphorylation of purified and reconstituted β_2-adrenergic receptors earlier (Hekman *et al.*, 1994). These findings suggest that the $G_{\beta\gamma}$-binding domain (possibly PH domain) of the GRKs is also a site for lipid-protein interaction. Thus, it seems that both lipids and G-proteins co-regulate the function of GRKs. Phosducin, on the other hand can be phosphorylated by PKA upon which the affinity for $\beta\gamma$ subunits decreases (Bauer *et al.*, 1992). These findings indicate that this system is quite complex containing various positive and negative feedback loops.

Also the existence of additional cDNA clones encoding novel β-arrestin-related proteins, termed β-arrestin2 and 3, have been reported (Attramadal *et al.*, 1992, Sterne-Marr, 1993). Questions concerning the receptor specificity of these various arrestins arise also for this family of proteins. Recently, Gurevich *et al.* (1995) have identified structural elements that contribute to arrestin binding and selectivity using truncated and chimeric arrestins, suggesting a common molecular mechanism involved in determining receptor binding selectivity. Apparently, GRK- and arrestin-mediated receptor desensitization reflects a general occuring mechanism with regard to the GPCRs. The extensive distribution of the different GRKs and arrestins, also in areas were no β_2-adrenergic receptors are located, supports this fact (Arriza *et al.*, 1992, Attramadal *et al.*, 1992).

As already mentioned, several GPCRs can also be phosphorylated by their respective effector kinases, PKA and PKC (see for references Lohse, 1993). PKA and PKC are respectively activated by cAMP and DAG, second messengers produced after activation of the adenylyl cyclase and phosphoinositide pathway respectively. Since PKA and PKC, may be activated upon stimulation of other receptor systems, they are considered to be responsible for several reports of the heterologous type of desensitization.

The region important for the heterologous type of short-term desensitization of the β_2-adrenergic receptor, mediated by PKA, appears to reside in the third intracellular loop and in the N-terminal part of the C-terminus. At these locations consensus sites for PKA (consensus sequence: XRRXSX, (X=no specific amino acid, R=arginine, S=serine), Kemp and Pearson, 1990) exist. Both regions, but in particular the third intracellular loop, appear to be involved in receptor-G_s coupling. Phosphorylation of these sites introduces a highly charged moiety into these regions leading to important alterations in the conformation of these regions. As for the phospholipase C-linked receptor systems, PKC consensus sites (consensus sequence: XRXXSXRX, Kemp and Pearson, 1990) were found to reside in particular in the third intracellular loop, a region important for G-protein coupling. Several of these receptors appear to undergo PKC-mediated desensitization (see for references Lohse 1993). Moreover, phorbol esters, activators of PKC, were found to phosphorylate and desensitize the β_2-adrenergic receptor (Sibley *et al.*, 1987). PKC and PKA were found to phosphorylate the β_2-adrenergic

receptor at the same site as their effects were not additive (Sibley *et al.*, 1987). These data indicate that phospholipase C-linked receptors may also contribute to some forms of heterologous desensitization of adenylyl cyclase-linked receptors.

The extent of PKA-mediated desensitization of the β_2-adrenergic receptor is comparable to the extent mediated by the GRK/β-arrestin mechanism. Yet, the agonist-induced PKA-mediated desensitization is considerably slower ($t_{1/2}$ ~ 2 min) than the GRK-mediated process ($t_{1/2}$ ~ 15 secs), but is more sensitive to low agonist concentrations (EC_{50} ~ 10 nM vs ~ 300 nM). It is therefore believed that the PKA-mediated desensitization may be a sensitive ubiquitous process occurring in response to low agonist concentrations, whereas GRK-mediated desensitization might occur at regions were high concentrations of neurotransmitters are to be found, e.g. synapses (Lohse *et al.*, 1990). In fact, the expression of the GRKs were shown to be high at post-synaptic locations (Arriza *et al.*, 1992, Attramadal *et al.*, 1992).

Thus, two different patterns of short-term desensitization appear to exist. The first involves a rapid strict homologous type of desensitization mediated by specific receptor kinases (GRKs). The heterologous desensitization is somewhat less rapid but much more sensitive to low agonist concentrations and is mediated by effector kinases such as PKA and PKC. In view of the reported heterogeneity of these kinases (Nishizuka, 1988) the complexity of the system is further enhanced. Yet, at this moment no information is available on the involvement of specific kinase isoforms.

Besides regulation at the level of the receptor, there is also evidence indicating that regulation may take place at post-receptor levels as well. In particular at the level of G-proteins, mechanisms affecting G-protein function and G-protein number can be distinguished. A small number of intracellular proteins (e.g. GAP-43, ras-GAP, phosducin) have been found to be responsible for altering G-protein function by activating or inhibiting G-protein-mediated signalling (see for more information references Lohse, 1993). Moreover, desensitization of GPCRs was found to induce phosphorylation or alterations in expression of G-protein subunits, thereby altering G-protein receptor function. Long-term activation of receptors which are positively coupled to adenylyl cyclase was found to be accompanied by a decrease of $G_{s\alpha}$ levels but increase in expression of $G_{i\alpha}$ (see for references Lohse, 1993, Mullaney *et al.*, 1993). For the receptors that are negatively coupled to adenyly cyclase and those coupled to phospholipase C, similar observations were made with regard to downregulation of respectively $G_{\alpha i}$ and $G_{\alpha q}$ (see for references Lohse, 1993, Mullaney *et al.*, 1993).

As already stated in section 1.1 the effector enzymes adenylyl cyclase and phospholipase C consist of a family of isoenzymes, which each appear to be differentially regulated (Cockcroft and Thomas, 1992, Iyengar, 1993, Tang and Gilman, 1992). Regulation of these isoenzymes may contribute to the overall phenomenon of receptor desensitization. $\beta\gamma$ subunits for example were shown to regulate some isoenzymes of the adenylyl cyclase and phospholipase C family (Iyengar, 1993, Fisher, 1995). Various isoenzymes were even found to be susceptible to cross-regulation. Activation of PKC or increases of the intracellular Ca^{2+} concentration, were

shown to regulate adenylyl cyclase activity either positively or negatively (see Gusovsky and Gutkind, 1991, Iyengar, 1993). Similarly, heterologous regulation of phospholipase C by acute activation of adenylyl cyclase-linked receptors has frequently been associated with an inhibition of receptor-stimulated phosphoinositide hydrolysis (Dickenson *et al.*, 1993), although enhancement of phosphoinositide hydrolysis has also been reported (see for references Fisher, 1995). The mechanism underlying the differential ability of cAMP to either inhibit or potentiate receptor-stimulated phosphoinositide hydrolysis remains to be determined.

Another class of proteins that may contribute to desensitization of the adenylyl cyclase-coupled receptors are the cAMP phosphodiesterases. At least five different isoenzyme families are now recognized, which appear to be differentially expressed, regulated and distributed (Beavo and Reifsnyder, 1990, Nicholson *et al.*, 1991). There is evidence that these phosphodiesterases can be rapidly regulated by cAMP- and Ca^{2+}/calmodulin-dependent protein kinases (see for references Nicholson *et al.*, 1991). A slower cAMP-mediated control of phosphodiesterase mRNA transcription has also been reported (see for references Nicholson *et al.*, 1991).

For the phospholipase C-linked receptors, desensitization or downregulation of the IP_3 receptor located at intracellular Ca^{2+} pool or ineffective Ca^{2+}-pump or Ca^{2+} release mechanisms due to prolonged agonist-exposure have been proposed to play a possible role in the process of receptor desensitization (see for references Wojcikiewicz *et al.*, 1993).

Receptor internalization

Besides an uncoupling of GPCRs from their respective G-proteins, agonists can also induce a translocation of receptors to intracellular sites. The inaccessibility of internalized receptors to hydrophilic ligands (e.g. the aminergic agonists), but accessibility to hydrophobic ligands, as well as the occurrence of receptors in fractions lighter than membrane fractions and disruption of internalization by specific inhibitors, suggest that sequestered receptors are internalized into intracellular endosomes (Hertel *et al.*, 1985, Maloteaux and Hermans, 1994, Von Zastrow and Kobilka, 1992, 1994). More direct evidence for such a hypothesis was given by means of immunofluorescence confocal microscopy, showing β_2-adrenoceptor immunoreactivity in intracellular vesicles and colocalization with transferrin receptors (Von Zastrow and Kobilka, 1992, 1994). Thereafter, other reports showed similar results for other GPCRs using either epitope-tagged GPCRs or antibodies raised against the N- or C-terminus (Garland *et al.*, 1994, Von Zastrow and Kobilka, 1993). Moreover, using similar techniques recently Mantyh *et al.* (1995) have found evidence for *in vivo* internalization of the substance P receptor in the rat striatum, suggesting that the observations made on the internalization and recycling of GPCRs in transfected cell lines *in vitro* is applicable *in vivo*.

At first it was thought that internalization was the major mechanism of receptor desensitization (Waldo *et al.*, 1983). However, internalization is a rather slow process

compared to the rapid phosphorylation and uncoupling of receptors. Moreover, the extent of receptor internalization can not explain the observed extent of desensitization (Lohse, 1990). Yet, internalization may be considered as a mechanism of receptor resensitization (Pippig *et al.*, 1995, Sibley *et al.*, 1986, Yu *et al.*, 1993). Sequestered receptors exhibit a decreased degree of phosphorylation as compared to surface receptors and vesicles are found to be enriched with phosphatases (Sibley *et al.*, 1986). The hypothesis regarding resensitization is further supported by the finding that prevention of internalization by sucrose or phenylarsine oxide, known as blockers of internalization, inhibit receptor resensitzation (Hertel *et al.*, 1985, Feldman *et al.*, 1986, Garland *et al.*, 1994, Palmer *et al.*, 1994, Pippig *et al.*, 1995, Yu *et al.*, 1993) Moreover, internalization-defective β_2-adrenergic receptors (Barak *et al.*, 1994, Yu *et al.*, 1993) do not recover from desensitization.

For the β_2-adrenergic receptors initial studies have shown that second messenger production, activation of the effector system or phosphorylation by PKA or GRKs do not seem to be prerequisites for agonist-induced internalization, as mutated receptors showing impaired G_s-coupling or cellular systems lacking the machinery to activate the G-protein mediated effector system or receptors lacking potential phosphorylation sites still show internalization upon agonist exposure (Campbell *et al.*, 1991, Hausdorff *et al.*, 1989, Lohse, 1990, Mahan *et al.*, 1985, Strader *et al.*, 1987). Yet, serine- and threonine-rich sequences in the cytopasmatic tail of the β_2-adrenergic receptor seem to play an important role in the process of internalization, as receptor mutants lacking these phosphorylation sites show impaired internalization (Barak *et al.*, 1994, Bouvier *et al.*, 1988, Campbell *et al.*, 1991, Hausdorff *et al.*, 1991). Internalization has also been reported for other GPCRs, including the α_1-adrenoceptors, the gastrin-releasing peptide, thyroptropin and angiotensin AT_{1A} and muscarinic receptors (Eva *et al.*, 1990, Benya *et al.*, 1993, Hunyady *et al.*, 1994, Nussenzveig *et al.*, 1993, Thomas *et al.*, 1995). For most receptors that couple to phospholipase C, the production of second messengers appears not to be a prerequisite (Laduron, 1994, Fisher, 1995, Slowiejko *et al.*, 1994). However, for the muscarinic m1 and gastrin-releasing peptide receptors intact receptor-G-protein coupling seems to be important (Benya *et al.*, 1994, Lameh *et al.*, 1992. Mutational analysis revealed that for the human m1 muscarinic receptor deletion of the small central region of the third intracellular loop is sufficient to impair internalization severely (Lameh *et al.*, 1992). Substitution of two amino acids, which are essential for G-protein coupling, resulted in an attenuation of the agonist-induced internalization of the gastrin-releasing peptide receptors, indicating that the gastin-releasing peptide receptor requires intact receptor-G-protein coupling (Benya *et al.*, 1994).

The C-terminal tail of many GPCRs appears to be an important structural determinant for receptor internalization. As stated before removal of serine/ threonines residues from the C-terminal tail of the β_2-adrenergic receptor prohibits receptor internalization (Barak *et al.*, 1994, Bouvier *et al.*, 1988, Campbell *et al.*, 1991, Hausdorff *et al.*, 1991). Moreover, truncation of the C-terminal tail of the thyrotropin-releasing hormone receptor and angiotensin II receptor

also resulted in reduction of internalization (Nussenzveig *et al.*, 1993, Thomas *et al.*, 1995). For the β_1-adrenergic receptor, a proline-rich region in the third intracellular loop, however, was found to induce conformational changes that might impair internalization, as removal of this region increased internalization (Green and Liggett, 1994). Insertion of this proline-rich region into the β_2-adrenergic receptor resulted in an impaired internalization. These findings indicate that different domains of the C-terminal tail can modulate receptor internalization in opposite directions. This observation is perfectly illustrated by the findings that the cytoplasmatic tail of the parathyroid hormone receptor contains regions that are implicated in induction and others in inhibition of internalization (Huang *et al.*, 1995).

Receptor downregulation

Downregulation is a slow process compared to the two previously described desensitization phenomena (Collins, 1991, Lohse, 1993). It commonly occurs after many hours and may not be maximal before 24 hrs continuous agonist exposure. The mechanism of downregulation remains unclear but probably involves both enhanced degradation of internalized receptors and decreased receptor synthesis, induced by changes in gene expression (Von Zastrow and Kobilka, 1992, Hadcock and Malbon, 1989). One distinguishes two different components in the process of β_2-adrenergic receptor downregulation, i.e. an agonist- and PKA-mediated (heterologous) component. S49 mouse lymphoma cells defective in post-receptor components (G_s, adenylyl cyclase or PKA) were shown to be still susceptible to β_2-adrenergic receptor downregulation, indicating an agonist-dependent mechanism (Allen *et al.*, 1989, Shear *et al.*, 1976, Su *et al.*, 1980). Defects in β_2-adrenoceptor-G_s coupling resulted in impaired receptor downregulation, suggesting the importance of receptor-G_s coupling in the process of receptor downregulation (Campbell *et al.*, 1991, Hadcock *et al.*, 1989, Mahan *et al.*, 1985, Shear *et al.*, 1976, Su *et al.*, 1980). Addition of exogenous cAMP or phosphodiesterase inhibitors could not overcome the observed impaired downregulation in these receptor mutants. Moreover, mutational analysis of the β_2-adrenergic receptor have shown that the palmitoylated cysteine at position 341 (Campbell *et al.*, 1991) and the N-terminal and C-terminal portions of the third intracellular loop (Campbell *et al.*, 1991) which are involved in G_s-coupling, were found to be important for β_2-adrenergic receptor downregulation as well. These findings show that either a G_s-mediated but PKA independent signal is required for receptor downregulation, or that receptor-G_s complexes are preferentially degraded. Moreover, it should be noted that while mutant receptors in these studies could be fully impaired in G_s-coupling, the downregulation was never fully blocked.

Besides the agonist-induced downregulation, cAMP analogues or forskolin were also shown to promote receptor β_2-adrenergic receptor downregulation, which is referred to as the PKA-dependent downregulation (Bouvier *et al.*, 1989). However, the rate at which β_2-adrenergic receptors are lost from the plasma membrane is considerably slower and often less

pronounced compared to the agonist-induced effects. Site-directed mutagenesis experiments have shown that phosphorylation of the β_2-adrenergic receptor at a site in the third intracellular loop by PKA may modestly enhance the rate of β_2-adrenergic receptor degradation (Bouvier *et al.*, 1989).

There are various reports describing downregulation of receptors that are negatively coupled to adenylyl cylcase and phospholipase C, including e.g. adenosine A_1, α_1-adrenergic and muscarinic receptors (see for references Fisher, 1995, Hadcock and Malbon, 1993). However, a distinction between agonist-induced and second messenger-induced downregulation of these receptors remain to be elucidated. In the neuroblastoma N1E-115 cell line and neuroblastoma/glioma hybrid cell line NG108-15, PKC stimulation with phorbolesters induces downregulation of respectively muscarinic (Liles *et al.*, 1986) and δ-opioid receptors (Gucker *et al.*, 1992), suggesting a role of PKC in the process of receptor downregulation. Evidence for the involvement of a PKC-dependent mechanism in the process of receptor downregulation of the gastrin-releasing peptide receptor was provided by mutational analysis, as removal of the PKC consensus site located in the C-terminal tail resulted in an attenuation of the agonist-induced downregulation (Benya *et al.*, 1995). Using site-directed mutagenesis, regions in the third intracellular loop of the muscarinic m1 receptor were found to play a role in the process of receptor downregulation (Shapiro and Nathanson, 1989).

Finally, the C-terminal tail of many GPCRs appears to be an important structural determinant for the process of receptor downregulation. Truncation or site-directed mutagenesis of serine, threonine or tyrosine residues of the C-terminal tail of for e.g. the β_2-adrenergic receptor and muscarine m3 receptor were shown to decrease the rate of receptor downregulation (Valiquette *et al.*, 1990, 1993, Cheung *et al.*, 1989, Bouvier *et al.*, 1989, Campbell *et al.*, 1991, Yang *et al.*, 1993). In addition, two isoforms of the somatostatin (mSSTR2A and mSSTR2B) (Vanetti *et al.*, 1993) and prostaglandin E_2 receptor ($EP_{3\alpha}$ and $EP_{3\beta}$) (Negishi *et al.*, 1993), which differ in their C-terminal tail, showed different patterns of receptor downregulation upon agonist exposure. Yet, the role of the C-terminal tail in receptor downregulation appears to be complex. For the avian β_1-receptor the C-terminal tail seems to prevent receptor downregulation as truncation of the receptor eliminates the insensitivity of the β_1-adrenergic receptor towards receptor downregulation (Hertel *et al.*, 1990, Parker *et al.*, 1991).

Generally, agonist-induced downregulation of GPCRs is accompanied by a decline of receptor mRNA levels (see for references review Hadcock and Malbon, 1993). The reduced levels of mRNA presumably contribute to the overall reduction in receptor number and responsiveness. Lowering of receptor mRNA levels serves to maintain the downregulated state by establishing a new steady-state level of receptors. As for the adenylyl cyclase-coupled receptors, the agonist-promoted downregulation of receptor mRNA appears to require PKA-activation although partial downregulation may also be induced by functional coupling of the receptor to G_s. In addition, an additional pathway exists for regulating receptor mRNA that

does not require elevated cAMP levels but only basal PKA activity (Hadcock and Malbon, 1993). For the phospholipase C-linked receptors the mechanism underlying decline of receptor mRNA is unclear, but for the α_{1B}-adrenergic receptor a decline in receptor mRNA levels may be induced by PKC (Izzo *et al.*, 1994).

Generally, cAMP- or Ca^{2+}-activated kinases are believed to phosphorylate factors involved in either repression of receptor gene transcription or selective degradation of receptor mRNA. In addition, transciption/translation of such factors may be induced (Port *et al.*, 1992). The mRNAs of several GPCRs are relatively short-lived: 30 min to 2 hrs (Collins, 1993), but breakdown can even be enhanced by agonist exposure. For the β_2-adrenergic receptor in DDT_1MF-2 cells, the β_2-adrenergic receptor expressed into CHW cells and the endothelin ET_B receptor in ROS17/2 rat osteosarcoma cells, the decline in receptor mRNA was ascribed to destabilization of the mRNA (Bouvier *et al.*, 1989, Hadcock *et al.*, 1989, Sakurai et al., 1992). Besides agonist-induced destabilization of mRNA a decrease of gene transcription may also contribute to the decrease of receptor mRNA levels. For the thyrotropin, luteinizing gonadotropin, platelet-activating factor, muscarinic m2 and m3 receptors, reduced rates of gene transcription have been proposed to be responsible for the lowered steady-state mRNA levels (Chau *et al.*, 1994, Fukamauchi *et al.*, 1993, Habecker and Nathanson, 1992, Saji *et al.*, 1992, Wang *et al.*, 1992).

In conclusion, events involving alterations in receptor function, intracellular translocation, downregulation, expression as well as alterations in the function and expression of G-proteins are found to contribute to the overall phenomenon of desensitization of GPCRs.

1.3.2 Regulation of histamine receptor function

As discussed in the previous sections receptor desensitization is regarded as one of the prominent mechanisms of receptor regulation. For the histamine receptors, such processes may become apparent under several pathophysiological conditions (e.g. asthmatic attack or allergic reaction), where histamine is released in large quantities, but might also occur under normal physiological conditions (see section 1.3.3).

Desensitization of the H_1 receptor has been reported *in vitro* in airway preparations (Anderson *et al.*, 1979, Brink *et al.*, 1982, Krzanowiski *et al.*, 1980, Leurs *et al.*, 1990a), intestinal smooth muscle preparations (Kenakin and Cook, 1979, Bielkiewicz and Cook, 1984, Hishinuma and Uchida, 1988, Leurs *et al.*, 1990b, Uchida and Hirano, 1983), cerebral preparations (Dillon-Carter and Chuang, 1989, Quach *et al.*, 1981, Bristow *et al.*, 1993) and several isolated cell systems (Brown *et al.*, 1986, Bristow and Zamani, 1993, Cowlen *et al.*, 1990, Dickenson and Hill, 1993, Lewis Baenzinger *et al.* 1981, McCreath *et al.*, 1994, McDonough *et al.*, 1988, Nakahata and Harden, 1987, Smit *et al.*, 1992, Zamani *et al.*, 1994). Excessive exposure of the histamine H_1 receptor, located on either airway, intestinal smooth muscle- or cerebral preparations of the guinea-pig, to a high concentration of histamine

was found to cause a time- and dose-dependent desensitization to subsequent stimulation (Bielkiewicz and Cook, 1984, Hishinuma and Uchida, 1988, Leurs, *et al.*, 1990c). Desensitization of histamine H_1 receptors present on cell lines such as BC3H-1-smooth muscle cells, human 1321N1 astrocytoma cells, human vascular endothelial cells, DDT_1MF-2 smooth muscle cells, human umbilical vein endothelial cells and human uterine HeLa cells was characterized by either an attenuated production of inositol phosphates or reduced capacity of the H_1 receptor to increase the intracelluar Ca^{2+} concentration (Brown *et al.*, 1986, Bristow and Zamani, 1993, Cowlen *et al.*, 1990, Dickenson and Hill, 1993, Lewis Baenzinger *et al.* 1981, McCreath *et al.*, 1994, McDonough *et al.*, 1988, Nakahata and Harden, 1987, Smit *et al.*, 1992, Zamani *et al.*, 1994).

Desensitization in canine and monkey tracheal strips *in vitro* and in human airways *in vivo* was suggested to result from a functional antagonism via a cyclooxygenase product (Anderson *et al.*, 1979, Krzanowiski *et al.*, 1980, Manning *et al.*, 1987). Yet, decrease of H_1 responsiveness in guinea-pig parenchymal lung strips could not be explained by the intervention of relaxing prostaglandines (Leurs *et al.* 1990a). However, contractions after KCl-induced membrane depolarization were reduced, suggesting that desensitization might affect the excitation-contraction coupling. In guinea-pig intestinal smooth muscle, however, KCl-induced membrane depolarization was unaltered, excluding dysfunction of the contractile apparatus in this preparation (Leurs *et al.*, 1990b).

In several cellular systems desensitization of the H_1 histamine receptor response appeared to be clearly restricted to the H_1 receptor, as other receptor mediated responses, e.g. muscarinergic, or direct activation of the G-protein by NaF remained unaffected (Cowlen *et al.*, 1990, Dickenson and Hill, 1993, Dillon Carter and Chuang, 1989, Hishinuma and Uchida, 1988, Leurs *et al.*, 1990c, McCreath *et al.*, 1994, Quach *et al.*, 1981, Smit *et al.*, 1992, Uchida and Hirano, 1983). The observed homologous desensitization implies a modulation at the level of the receptor. Possible modifications that might be responsible for the observed homologous desensitization are modulation of receptor binding characteristics (e.g. agonist affinity), attenuated receptor-G-protein coupling or loss of receptors.

Quach *et al.* (1981) explained the observed desensitization of the H_1 receptor-mediated glycogen breakdown in mouse cerebral cortex by a small reduction of the [^{3}H]-mepyramine binding sites. Yet, the loss of receptors could not entirely explain the reduced receptor activity. Moreover, in the guinea-pig intestine both maximal binding capacity and binding affinity of histamine for the histamine H_1 receptor remained unaltered (Leurs *et al.*, 1991c). In addition, in HeLa cells the number of H_1 receptors measured as [^{3}H]-mepyramine binding sites did not alter after short-term histamine pretreatment (Smit *et al.*, 1992).

Investigation of the effect of short-term and in particular long-term histamine-treatment on H_1 receptor density has been hampered by the lack of suitable model systems, possessing a reasonable density of H_1 receptors. The recent cloning of the genes encoding the H_1 receptor

(De Backer *et al.*, 1993, Fujimoto *et al.*, 1993, Fukui *et al.*, 1994, Horio *et al.*, 1993, Moguilevsky *et al.*, 1994, Traiffort *et al.*, 1994) permits the generation of cell lines expressing sufficient amounts of H_1 receptors (Iredale *et al.*, 1993, Leurs *et al.*, 1994c). Chapter 3 of this thesis describes the short-term, and long-term desensitization of the human H_1 receptor expressed in Chines Hamster Ovary (CHO) cells.

In contrast to the frequent reports on histamine H_1 receptor desensitization, relatively little information is available on histamine H_2 receptor desensitization. Desensitization of the histamine H_2 receptor has been described in HL-60 cells and was found to be mainly homologous, although in some experiments prostaglandin (PGE_2) responses were also affected, indicating a small heterologous component (Johnson and Sawutz, 1984). A phospholipase A_2 inhibitor, reported to be effective against β-adrenergic receptor desensitization in astrocytoma cells, inhibited the heterologous induced desensitization in HL-60 cells (Mallorga *et al.*, 1980). The mechanism of action of this inhibitor is however unclear. Desensitization in HL-60 cells could not be explained by a loss of H_2 receptors nor by the induction of phosphodiesterase activity. However, in mononuclear leukocytes, histamine caused a heterologous desensitization of the H_2 receptor, β-adrenergic and prostaglandin E_2 receptor, which could be explained by enhancement of phosphodiesterase activity (Chan *et al.*, 1982, Holden *et al.*, 1987).

In clonal cytolic T lymphocytes histamine was reported to induce an homologous desensitization of the H_2 receptor as no effect on isoproterenol, PGE_2 or forskolin stimulated cAMP formation was observed (Schreurs *et al.*, 1984). The half time of desensitization was less than 10 min, possibly indicating receptor-cyclase uncoupling rather than receptor internalisation or downregulation. In addition, in the human gastric cancer cell line HGT-1 H_2 receptors have also been reported to be desensitized upon exposure to histamine with a half time of 20 mins (Prost *et al.*, 1984). Recent reports, however, have demonstrated the existence of a histamine H_2 receptor on HL-60 and HGT-1 cells with a pharmacological profile distinct from the commonly described H_2 receptor (Burde *et al.*, 1989, Mitsuhashi *et al.*, 1989, Reyl-Desmars *et al.*, 1991, Seifert *et al.*, 1992). The human monocytic cell line U937, appeared to express H_2 receptors pharmacological identical to the H_2 receptor located on the right atrium of the guinea-pig and was therefore chosen as model system for the investigation of H_2 receptor desensitization in our laboratory (Smit *et al.*, 1994, Chapter 4). The H_2 receptors present on this cell line were found to be susceptible to homologous receptor desensitization as the β-adrenoceptor-mediated effect remained unaffected (Smit *et al.*, 1994, Chapter 4). Homologous desensitization of the H_2 receptor has also been observed in the human gastric carcinoma cell line MKN-45 (Arima *et al.*, 1993). The histamine-induced desensitization in MKN-45 cells did not appear to be associated with a reduction in [^{3}H]-tiotidine binding. Fukushima *et al.*, (1993) on the other hand, showed a 25% decrease of [^{3}H]-tiotidine binding in intact and membranes of CHO cells, expressing the canine H_2 receptor, treated with 10 μM histamine for 1 hr, a condition where H_2 receptors were found to

be desensitized. In contrast, histamine treatment induced no apparent change in H_2 receptor level as assessed by immunoblotting with a antipeptide antibody against the C-terminal tail sequence of the receptor. Moreover, if one takes into account the technical difficulties associated with [^{3}H]-tiotidine binding assays (Garbarg *et al.*, 1992), the validity of the observed decrease of only 25% should be questioned. The introduction of a highly sensitive H_2 antagonist [^{125}I]-aminopotentidine (Ruat *et al.* 1990b) may give more insight in the effect of histamine-induced desensitization on H_2 receptor expression.

As already discussed for the H_1 receptor, binding studies with the commonly used cellular systems are often hampered by the low expression of H_2 receptors. The elucidation of DNA sequences encoding the histamine H_2 receptor protein (Gantz *et al.*, 1991a, Gantz *et al.*, 1991b, Ruat *et al.*, 1991) has made it possible to generate stable cell lines expressing high densities of H_2 receptor protein (Fukumashi *et al.*, 1993, Leurs *et al.*, 1994a, Traiffort, 1992b). These model systems enable for the first time investigation of long-term regulatory mechanisms of the H_2 receptor. In Chapters 5 to 9, the effects of long-term exposure of H_2 receptors to H_2 agonist and antagonists are studied and discussed.

As shown in section 1.3.1 desensitization is thought to be associated with post-translational modification of the receptor protein by phosphorylation (Hausdorff *et al.* 1990, Huganir and Greengard, 1990, Lefkowitz *et al.*, 1990). Kinases, such as PKA and PKC are responsible for the heterologous type of receptor desensitization, whereas GRKs seem to play crucial roles in the mechanism underlying homologous desensitization (Huganir and Greengard, 1990, Lefkowitz *et al.*, 1990, Palczewski and Benovic, 1991, see section 1.3.1).

Indeed, desensitization of the histamine H_1 receptor appears also to be regulated by kinases. In various systems PKC has been found to account for a negative feedback on the histamine receptor-mediated response (Cowlen *et al.*, 1990, Dickenson and Hill, 1993, Dillon-Carter and Chuang, 1989, Fukui *et al.*, 1991, Leurs *et al.*, 1990c, 1991c, McCreath *et al.*, 1994, McDonough *et al.*, 1988, Tilly *et al.*, 1990a,b, Smit *et al.*, 1992, Volpi and Berlin, 1988). In some cellular systems exposure of histamine H_1 receptors to other stimulating agents, which activate receptors coupled to phospholipase C, resulted also in an attenuation of H_1 receptor-mediated responses, referred to as heterologous desensitization (Hishinuma and Uchida, 1988, Leurs *et al.*, 1990b, McDonough *et al.*, 1988). This type of desensitization is most likely caused by a commonly activated molecular entity such as the G protein or alterations at the level of the intracellular Ca^{2+} pool.

Evidence for the possible involvement of a specific kinase (GRK) in the process of H_1 receptor desensitization has been obtained by experiments using Ca^{2+} fluorescence microscopy and video digital imaging in human HeLa cells (Smit *et al.*, 1992, see Chapter 2). In HeLa cells histamine induces an immediate H_1 receptor mediated biphasic elevation of the intracellular Ca^{2+} concentration. Desensitization, characterized by an impaired release and influx component of the Ca^{2+} response, was monitored when the HeLa cells were repeatedly exposed to histamine. The histamine induced release component appeared to be reduced in a

selective manner, as neither the caffeine- nor the ATP-induced Ca^{2+} release components were found to be affected. The observed inhibition of the influx component can be explained by the involvement of PKC, as activation of PKC by a phorbolester both affected the histamine- and ATP-induced Ca^{2+} response. Yet, inhibition of the Ca^{2+} release component was still observed when PKC activity was eliminated, suggesting the involvement of another regulatory mechanism, possibly a specific receptor kinase (GRK). Based on these experimental data it was suggested that short-term desensitization of the histamine H_1 receptor in HeLa cells evolves from two different processes: a PKC dependent pathway, and a PKC independent pathway (Smit *et al.*, 1992).

Also in guinea-pig lung tissue evidence has been obtained for the involvement of a PKC-independent mechanism of receptor desensitization (Leurs *et al.*, 1992) The non-selective kinase inhibitor H-7 was found to attenuate H_1 receptor desensitization, whereas selective PKC inhibitors were ineffective. Moreover, H-7 was found to attenuate β-adrenoceptor-induced translocation of a GRK-like enzyme from the cytosol to the membrane (Garcia-Higuerea and Mayor, 1992). Thus, it is tempting to speculate that H-7 interferes with a specific kinase, inhibiting its translocation to the membrane, leading to H_1 receptor desensitization.

Moreover, in human umbilical vein endothelial cells and DDT_1MF-2 cells the histamine-induced desensitization appeared to be homologous and insensitive to PKC inhibitors, excluding a role for PKC and suggesting the involvement of a specific mechanism in this process (Dickenson and Hill, 1993, McCreath *et al.*, 1994). Other kinase inhibitors such as Ca^{2+}/calmodulin or cGMP-dependent kinase inhibitors failed also to modify the histamine-induced desensitization of H_1 receptor-mediated responses in human umbilical vein endothelial cells (McCreath *et al.*, 1994).

For the H_2 receptor there is no direct evidence for the involvement of GRKs, but the observed homologous desensitiation in U937 and MKN-45 cells suggest such an involvement (Arima *et al.*, 1993, Smit *et al.*, 1994).

Taken together, it is conceivable that agonist-induced, short-term desensitization of the histamine receptors involves activation of a GRK, analogous to the proposed mechanism for desensitization of the β-adrenoceptor system. Recently, the genes encoding the histamine H_1 and H_2 receptor were cloned (see section 1.2.1). The primary sequence of both the histamine H_1 and H_2 receptor reveals the existence of several serines and threonines in the third intracellular loop and cytoplasmatic tail, which may serve as potential phosphorylation sites in the process of desensitization (Fig. 2 and 3). Moreover, these potential phosphorylation sites may also be involved in the process of receptor internalization or downregulation. Besides the serines and threonines, other structural features, which were reported to be important for regulation of the β_2-adrenergic receptor, such as various conserved tyrosine residues, are also present in the H_2 receptor. The histamine H_1 receptor contains several PKC sites and also a potential PKA site (Fig. 2). The histamine H_2, on the other hand, does not seem to possess

potential PKA sites (Fig. 3). However, this does not rule out the possibility that the H_2 receptor is phosphorylated by PKA, since e.g. nitric oxide synthase was found to be phosphorylated by PKC despite the absence of a potential PKC site (Bredt *et al.*, 1991). Clearly, the availibility of the genes encoding the histamine H_1 and H_2 receptor offers new possibilities with regard to the elucidation of the mechanism underlying receptor desensitization (Chapters 3, 5 to 9).

1.3.3 Clinical aspects of receptor desensitization and implications for drug therapy

As shown in previous sections (1.3.1, 1.3.2) receptor regulation is functionally diverse and in many cases rather complex. Transcriptional, post-transcriptional and post-translational mechanisms are found to contribute to the overall control of receptor expression and cellular responsiveness.

Under pathophysiological conditions such mechanisms may become apparent. In e.g. patients suffering from congestive heart failure, loss of heart function is associated with increased levels of noradrenaline, decreased neuronal adrenergic uptake and a decreased β-adrenoceptor function (Homcy et al., 1991, Hammond, 1993, Stiles, 1991). The latter can be explained by a decreased sensitivity, due to receptor uncoupling and by a reduction in the number of myocardial β_1-adrenoceptors (Homcy *et al.*, 1991, Hammond, 1993, Stiles, 1991). First, it was assumed that the hormone sensitive adenylyl cyclase system of the heart in patients with congestive heart failure is less responsive to catecholamine stimulation in terms of cAMP generation. However, stimulatory agonists such as histamine and forskolin demonstrated unaltered activity (see for references Ungerer *et al.*, 1993). In view of these findings inotropic H_2 agonists are considered as potential drugs which might be used in treatment of patients with congestive heart failure (Felix *et al.*, 1991). Moreover, Ungerer *et al.* have shown that the diminished response to β-adrenergic agonist can be ascribed to increased expression of GRKs (Ungerer *et al.*, 1993). In addition, it has been found that the level of the inhibitory G-protein G_i is increased in myocardial tissue from patients with congestive heart failure (Feldman *et al.*, 1988, Neuman *et al.*, 1988). Increase in GRK2 as well as β-arrestin levels, has also been reported after chronic morphine administration in the rat locus coeruleus and not in other brain areas, suggesting that elevation of both entities may contribute to opioid-receptor tolerance which is known to occur in this brain region (Terwilliger *et al.*, 1994). In view of the potential use of H_2 agonists in the treatment of patients with congestive heart failure it is important to determine whether the H_2 receptor is susceptible to desensitization mechanisms as found for the β_2-adrenergic receptor (Chapter 4).

For the histamine receptors there are various reports describing *in vivo* desensitization of histamine receptor function (Antol *et al.*, 1988, Manning *et al.*, 1987, Poulakos and Gertner, 1986). The mechanism underlying the observed desensitization, however, is still unclear. There are also various reports describing dynamic regulation of H_1 receptor density *in vivo*

(Iinuma *et al.*, 1993, Martinez-Mir *et al.*, 1993, Nakai *et al.*, 1991, Yanai *et al.*, 1992). Upon aging the number of H_1 receptors was shown to decrease (Yanai *et al.*, 1992). Changes of H_1 receptor density have also been reported in the electric focii in the temporal cortex of epileptic patients (upregulation H_1 receptors) and in the frontal cortex of patients with chronic schizophrenia (downregulation H_1 receptors) (Iinuma *et al.*, 1993, Nakai *et al.*, 1991). Similarly, the H_2 receptor density was found to be altered in brain tissue of patients suffering from Huntington's Chorea disease (downregulation H_2 receptors) (Martinez-Mir *et al.*, 1993). These examples indicate that H_1 and H_2 receptor expression is susceptible to regulatory changes under physiological and pathophysiological conditions.

Desensitization has also been observed during drug therapy (Brodde *et al.*, 1990, Motomura *et al.*,1990, Ghosh *et al.*, 1991, Homcy *et al.*, 1991, Stiles, 1991). Healthy volunteers receiving prolonged treatment with β_1 and β_2 agonists display reduced β-adrenoceptor function (Brodde *et al.*, 1990). There are also reports which show altered GPCR function after antagonist treatment. Upregulation of β1-, β_2-adrenergic and opoid receptors after chronic antagonist treatment have been reported (Boudoulas *et al.*, 1977, Elfellah *et al.*, 1989, Motomura *et al.*, 1990, Yoburn *et al.*, 1994). Patients with severe heart failure have been beneficially treated with low doses of selective β_1-antagonists (Heilbrunn et al., 1989). The theoretical rationale for such treatment is that β_1-antagonists can increase β-adrenergic density, and thus may restore the previously reduced β-adrenergic density in these patients. As for the H_1 receptors, long-term (1 week) treatment of patients with mild atopic asthma with the H_1 antagonist cetirizine resulted in a loss of effect of cetirizine itself on histamine-induced bronchoconstriction, compared to when administerd only once (Ghosh *et al.*, 1991). These observations were suggested to result from increased sensitivity of H_1 receptors, caused by a possible upregulation of H_1 receptors. Based on the results from a study in which patients were chronically treated with the H_1 antagonist chlorpheniramine similar suggestions were made (Taylor *et al,*. 1985). Similarly, H_2 antagonists were shown to lose their antisecretory effects in patients after prolonged dosing, which might be explained by a possible upregulation of H_2 receptors (Bertaccini *et al.*, 1992, Merki *et al.*, 1994, see Chapter 9). Moreover, chronic treatment with the H_2 antagonists ranitidine resulted in increased parietal cell sensitivity in consious cats and abrupt withdrawal of histamine H_2 receptor blockade led to intragastric hyperacidicity (Nwokolo *et al.*, 1991, Coruzzi *et al.*, 1989), which also may be ascribed to an increase of H_2 receptor density (see Chapter 9). Schizophrenic patients receiving chronic neuroleptic treatment display increased H_2 receptor densities in the globus palllidus (Martinez-Mir *et al.*, 1993). As some neuroleptics show considerable affinity for the human H_2 receptors (Traiffort *et al.*, 1992b, Leurs *et al.*, 1994a), it is conceivable that neuroleptics may modulate H_2 receptor expression *in vivo*.

It is evident that the occurence of desensitization after agonist treatment and observed changes in GPCR function after antagonist treatment may have implications for the

development of efficient drug therapy. Approximately 50 to 60 % of all clinical relevant drugs exert their action via GPCRs (Guderman *et al.*, 1995). In view of the wide use of H_1 and H_2 antagonists in the treatment of allergies (Janssens, 1993) and gastric ulcers respectively (Bertaccini and Corruzzi, 1992, Deakin and Williams, 1992) and the putative therapeutical application of H_2 receptor agonists in patients suffering from congestive heart failure (Felix *et al.*, 1991), a detailed study of the mechanisms underlying the regulation of histamine H_1 and H_2 receptor expression is indispensable (see 1.4 Aim of thesis).

Effective drug therapy requires detailed knowledge of the phenomena of receptor regulation. The degree and the development of regulatory phenomena depend on the presence and expression level of the molecular entities (e.g. GRKs, arrestins) in the cellular environment of that receptor. As such, regulation of one receptor may differ from cell to cell. Receptor regulation may even vary within a population, as receptor mutations were found to explain phenotypic variations leading to altered receptor regulation (Reihsaus *et al.*, 1993, Green *et al.*, 1994). Recently, Reihsaus *et al.* (1993) have identified in the general population different polymorphic forms of the β_2-adrenergic receptor, including three forms that are compromized in the N-terminus of the receptor, encompassing a single mutation. Although there is no clear idea how this extracellular part of the receptor can influence receptor regulation, *in vitro* studies have indicated that these polymorphisms of the β_2-adrenergic receptor impart distinct patterns of agonist-promoted downregulation; two promoting downregulation, another inhibiting downregulation (Green *et al.*, 1994). As such, these polymorphisms, occurring at frequenties ranging from ~10% to ~50 %, may be responsible for the heterogeneity between individuals in β_2-adrenergic receptor expression and function. Reihsaus *et al.* even suggested that these natural occuring polymorphisms may explain the clinical status of asthmatic patients (Reihsaus *et al.* 1993). It is therefore tempting to speculate that hypersensitivity of asthmatic patients for histamine is caused by polymorphic variations of the histamine H_1 receptor within the population.

All the molecular entities involved in the processes of receptor regulation may form possible targets for interference to prevent undesired receptor regulation. Potential strategies to prevent receptor desensitization, include inhibitors of e.g. GRKs or arrestins, as these molecular entities were shown to contain domains that comprise selectivity (Inglese *et al.*, 1992, Haga *et al.*, 1994, Gurevich *et al.*, 1995). Moreover, *in vitro* studies have shown that a synthetic peptide corresponding to the last 28 amino acid residues from the C-terminal region of βARK was able to block βγ activation of GRK2/3, and may therefore provide a useful strategy for the inhibition of the enzyme (Koch *et al.*, 1993). Other possible strategies may be envisaged so that in the future effective therapy consists of a multitargeting drug approach, in order to eliminate regulatory changes of GPCR function after agonist or antagonist medication.

1.4 Aim of thesis

The rapid regulation of receptor function appears to be an integral part of the transmembrane signalling of many GPCRs (section 1.3). The endogenous compound histamine is one of the mediators which is frequently released in inflammatory processes, leading to an instantaneous increase of the histamine concentration. It is therefore conceivable that histaminergic receptor responses may be affected and become desensitized. This regulatory process may serve as a protective mechanism, aiming to maintain a certain degree of intercellular communication. Desensitization of the implicated histamine receptor system may thus be favoured. In contrast, in tissues where histamine is essential for normal functioning desensitization should be reduced. Moreover, in view of the widespread use of H_1 (Janssens, 1993) and H_2 (Bertaccini and Corruzzi, 1992, Deakin and Williams, 1992) antagonists and proposed use of H_2 agonists (Felix *et al.*, 1991) and H_3 receptor ligands (Leurs *et al.*, 1995) detailed knowledge of the regulation of the histamine receptors is important for development of effective drug therapy. Besides, there are various reports describing altered density of histamine receptors *in vivo*, observed under pathophysiological but also physiological conditions (see section 1.3.2). Understanding receptor regulation might give new insights in the respective (patho) physiological conditions, but might also create new strategies for modulating receptor desensitization. All molecular entities involved in the process of desensitization represent possible targets for therapeutic intervention to modify the process of desensitization and thus cellular communication.

In this thesis the dynamic regulation of the histamine H_1 and H_2 receptor has been investigated. Previous studies in our laboratory have shown that the histamine H_1 receptor located on guinea-pig lung tissue and small intestine is rapidly desensitized upon histamine exposure (Leurs *et al.*, 1990a, b, 1991a, b, c, 1992). However, the use of these tissue preparations was limited as large quantities of material for radioligand binding studies and second messenger production were required. In addition, these preparations do not represent a homogeneous population of cells and histamine receptors. In our study we used cell lines expressing endogenous H_1 and H_2 receptors as a model system to examine the phenomenon of short-term receptor desensitization (Chapter 2 and 4). For the H_1 receptor we used the HeLa cell line, a human uterine carcinoma cell line (Chapter 2), and for the H_2 receptor the U937, a monocytic cell line (Chapter 4). Due to the low expression of histamine receptors in these cellular systems we subsequently used a transfected cell line (Chinese Hamster Ovary (CHO) cells) expressing the recently cloned human H_1 and human and rat H_2 receptor to examine long-term desensitization (Chapter 3 and 5). The pharmacological characterization of the human H_1 and H_2 receptor expressed in CHO cells is described in Chapters 3 and 7 respectively. Long-term receptor desensitization (downregulation) of the histamine H_1 and H_2 receptor was studied in Chapters 3 and 5. H_2 receptor mutants were generated to examine the biochemical mechanisms underlying H_2 receptor regulation and to determine the structural requirements for

H_2 receptor downregulation (Chapter 5, 6). In order to visualize the process of H_2 receptor internalization, epitope-tagged human and rat H_2 receptor were used and expressed in human embryonal kidney cells (HEK293) cells (Chapter 8). Besides the examination of short-term and long-term desensitization of the histamine H_1 and H_2 receptors, the long-term effects of H_2 antagonists on H_2 receptor expression were examined and discussed in Chapter 9.

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Chapter 2

Short-term desensitization of the histamine H_1 receptor in human HeLa cells: involvement of protein kinase C dependent and independent pathways

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In this study we have investigated the effects of short-term exposure of cells to histamine on the subsequent H_1 receptor responsiveness in HeLa cells, using Ca^{2+} fluorescence microscopy. In HeLa cells histamine (100 μM) induces an immediate H_1 receptor mediated biphasic elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (basal $[Ca^{2+}]_i$: 81 ± 30 nM, histamine-induced Ca^{2+} response: first phase: 1135 ± 79 nM; second phase: 601 ± 52 nM, n = 11). The histamine H_1 receptors on HeLa cells are readily susceptible to desensitization since repetitive exposure of the same group of cells to histamine (100 μM) markedly affected the release and influx component of the induced Ca^{2+} response (second application of histamine: first phase: 590 ± 92 nM, second phase: 279 ± 47 nM; third application of histamine: first phase: 454 ± 127 nM, second phase: 240 ± 45 nM, n = 6).

The influx component of the histamine-induced Ca^{2+} response appeared to be non-selectively reduced as also the second phase of the ATP response (50 μM) was significantly reduced after desensitization with histamine (control cells: 516 ± 33 nM; desensitized cells: 331 ± 96, n = 4). As activation of protein kinase C (PKC) by PMA was found to inhibit the histamine as well as ATP-induced Ca^{2+} response in a dose dependent manner, PKC seems to be responsible for the observed inhibition of the Ca^{2+} influx component. Moreover, in PKC downregulated cells the second phase of the histamine induced Ca^{2+} response was elevated, indicating the involvement of PKC in the negative feedback on the Ca^{2+} influx (control cells: second phase: 602 ± 52 nM; PKC downregulated cells: second phase: 837 ± 100 nM, n = 6).

The release component of the histamine-induced Ca^{2+} response appeared to be selectively reduced as neither the release component of the ATP response (50 μM) nor the caffeine-(3 mM) induced Ca^{2+} release were found to be affected by desensitization with 100 μM histamine. The data indicate the existence of homologous desensitization. The homologous desensitization of H_1 receptor responsiveness was still observed in PKC downregulated cells, implying the rapid activation of a regulatory mechanism other than PKC.

Based on our experimental data we suggest that short-term desensitization of the histamine H_1 receptor evolves from two different processes: a selective reduction of the histamine-induced Ca^{2+} release, mediated by a PKC independent pathway, and a non-selective inhibition of the receptor mediated Ca^{2+} influx activated by a PKC dependent pathway.

Introduction

G-protein coupled receptors (GPCRs) show frequently a dynamic regulation of receptor function. In response to various conditions receptor functionality can either be increased or diminished by cellular systems. Such a dynamic regulation of receptor responsiveness is not only observed under in vitro conditions but can also occur in vivo (Homcy *et al.*, 1991, Manning *et al.*, 1987) and has been suggested to have serious consequences for drug therapy (Brodde *et al.*, 1990, Ghosh *et al.*, 1991, Homcy *et al.*, 1991, Motomura *et al.*, 1990, Stiles *et al.*, 1991).

Desensitization is regarded as one of the prominent mechanisms of receptor regulation. This process is characterized by a loss of receptor responsiveness, despite the continuous presence of a high concentration of a stimulus (Hausdorff *et al.*, 1990, Huganir and Greengard, 1990, Lefkowitz *et al.*, 1990). This process might become apparent during drug therapy (Brodde *et al.*, 1990), but plays also a dominant role in several pathophysiological conditions (Homcy *et al.*, 1991, Stiles *et al.*, 1991).

In certain pathologies, e.g. an allergic reaction or asthmatic attack, the biogenic amine histamine is released in high quantities from either mast cells or basophils, resulting in an immediate, local increase of the histamine concentration. Thus, histamine receptors located near these sites are prone to be affected, which might result in receptor desensitization. To date, the process of desensitization of the H_1 and H_2 receptor has been reported in e.g. smooth muscle preparations (Anderson *et al.*, 1979, Bielkiewicz and Cook, 1984, Hishinuma and Uchida 1988, Kenakin and Cook, 1979, Leurs *et al.*, 1990, 1991b) and various isolated cell systems (Brown *et al.*, 1986, Cowlen *et al.*, 1990, Lewis Baenzinger *et al.*, 1981, McDonough *et al.*, 1988, Nakahata and Harden, 1987, Sawutz *et al.*, 1984). In earlier studies performed in our laboratory desensitization of the histamine H_1 receptor was suggested to be homologous and caused by an alteration at the level of the histamine H_1 receptor (Leurs *et al.*, 1990, 1991b). These findings were based on functional studies, measurements of inositol phosphate production and radioligand binding studies (Leurs *et al.*, 1990b, 1991). Nevertheless, the use of smooth muscle preparations has its limitations, since large quantities of material are needed for measurements of inositol phosphate production or radioligand binding studies. Moreover, since these preparations do not represent a homogeneous population of cells and histamine receptors, the use of these preparations for detailed mechanistic investigation is rather limited. These considerations prompted us to search for a well defined cellular system.

In the present study HeLa cells, a human uterine carcinoma cell line, was chosen as a model system to study the process of histamine H_1 receptor desensitization. Previous work from various laboratories has already revealed several characteristics with respect to the histamine H_1 receptor. Production of inositol phosphates, changes in ion permeability and Ca^{2+} signalling have been well delineated in this cell line after activation of the histamine H_1 receptor (Bristow *et al.*, 1991, Hazama *et al.*, 1985, Raymond *et al.*, 1991, Sauvé *et al.*, 1987, 1990, 1991, Tilly *et al.*, 1990a, b, c, Volpi and Berlin, 1988). Since these cells appear to possess a reasonable number of [^{3}H]-mepyramine binding sites (Bristow and Young, 1991), these cells might be suited to investigate H_1 receptor regulation.

The present study was undertaken in order to examine the effects of short-term exposure of HeLa cells to histamine on the subsequent H_1 receptor responsiveness. As parameter for receptor activation we used the increase in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$); changes in $[Ca^{2+}]_i$ were measured using fluorescence microscopy. Major advantages of this technique are the high sensitivity of this system and the ability to monitor direct changes in $[Ca^{2+}]_i$ upon

receptor activation in a group of 6 to 8 cells. In our experimental set up, changes in $[Ca^{2+}]_i$ induced upon second addition of a stimulus can be monitored in the same group of cells. This feature is especially important with regard to the agonist-induced desensitization. After an initial characterization of the H_1 receptor desensitization some experiments were performed to elucidate the underlying mechanism of this process.

Materials and methods

Cell culture Human HeLa cells were cultured at 37 °C in a humified atmosphere with 7.5 % CO_2 in Dulbelco's modified Eagle's medium supplemented with 7.5 % heat-inactivated fetal-calf serum (Integro, The Netherlands), 2 mM L-glutamine, 10 IU /ml penicilin and 10 µg/ml streptomycin (Flow Labarotories). The cells were passaged twice a week, using trypsine (0.05 %)-EDTA (0.2 mg/ml) (Flow laboratories) and grown to sub-confluency for the fluorescence measurements.

Measurements of $[Ca^{2+}]_i$ HeLa monolayers, grown on glass coverslips, were loaded with 10 µM fura-2-AM ester in a HBS buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 0.2 % BSA and 10 mM Hepes (pH 7.4)) for 30 mins at 37 °C . Ca^{2+}-dependent fura-2 fluorescence was monitored at an emission wavelength of 510 nm with an excitation wavelength of 340 nm and 380 nm (Grynkiewicz et al. 1985). Fluorescence measurements were carried out at 33 °C in HBS buffer using a fluorescence microscope (Leitz orthoplan, Ernst Leitz GHMB Wetzlar, Germany) and a SPEX dual wavelength fluorimeter as excitation source (SPEX Industries Inc., N.Y., U.S.A.). For Ca^{2+} measurements 6 to 8 cells were selected in all measurements. HeLa cells were washed every 5 mins by completely exchanging the medium by rapid perfusion during a period of 10 mins. Exposure of cells to a stimulus lasted for a period of 5 mins. Thereafter, cells were washed rapidly three times and were exposed to another stimulus 5 mins later. The second phase was recorded after 30 secs of receptor activation with the indicated drug. The background, largely due to autofluorescence, was measured prior to fura-2-AM loading and was corrected for in the calculation. Minimal fluorescence (F_{min}) was achieved by complexing calcium with 6 mM EGTA in Ca^{2+}-free HBS buffer (pH 8.0) and maximal fluorescence (F_{max}) was measured by permeabilization of cells with 3 µM ionomycin.

Chemicals Histamine dihydrochloride, methacholine chloride, bradykinin, phorbol-12-myristate-13-acetate, ionomycin and ATP (sodium salt) were obtained from Sigma Chemical Company Ltd. (St. Louis, MO, U.S.A.), fura-2-acetoxymethylester (fura-2-AM) was from Molecular Probes (Eugene, OR, U.S.A). The enantiomers of cicletanine were kindly donated by the Institute of Henri Beaufour (France).

Statistical analysis All data shown are expressed as mean ± s.e.mean of at least four independent experiments. Statistical analysis was carried out by Student's t-test. P-values < 0.05 were considered to indicate a significant difference.

Results

The histamine-induced calcium response in HeLa cells

In HeLa cells 100 µM histamine induced an immediate biphasic elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as measured by fura-2 fluorescence microscopy (Fig. 1A). This response was characterised by a rapid transient rise in $[Ca^{2+}]_i$, followed by a sustained increase in $[Ca^{2+}]_i$ and lasted until the agonist was removed (Fig. 1). The basal level of $[Ca^{2+}]_i$ in Hela cells was 81 ± 30 nM (n = 11), whereas after application of 100 µM histamine $[Ca^{2+}]_i$ was rapidly elevated to 1135 ± 79 nM (first phase, n = 11). After this initial rapid rise the $[Ca^{2+}]_i$ decreased to an elevated level of 601 ± 52 nM (second phase, n = 11) after 30 secs of receptor activation with histamine. The initial rise is largely dependent on the release of intracellular Ca^{2+}, whereas the second phase depends on the influx of extracellular Ca^{2+} (Tilly *et al.*, 1990b). As can be seen in Fig 1B the elevation of $[Ca^{2+}]_i$ by 100 µM histamine was

effectively blocked by 1 μM (-)-cicletanine, an H_1 receptor antagonist (Schoeffter *et al.*, 1987). The enantiomer (+)-cicletanine (1 μM), which is not active as an H_1 antagonist (Schoeffter *et al.*, 1987) did not affect the responsiveness of HeLa cells to histamine (Fig. 1C). These data clearly indicate the involvement of the histamine H_1 receptor in the response to histamine.

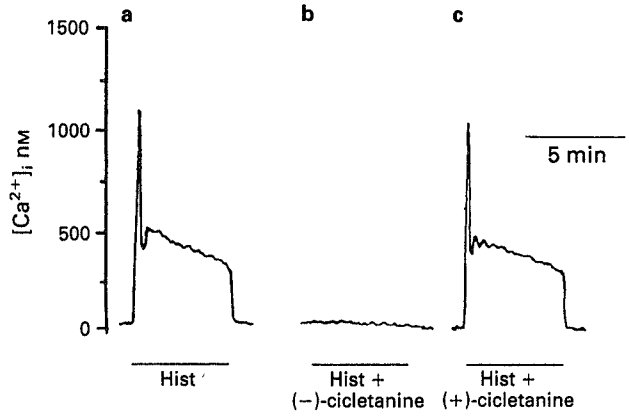

Fig. 1. Intracellular free Ca^{2+} transients induced by histamine (His, 100 μM) (a), histamine (100 μM) in the presence of (-)-cicletanine (1 μM) (b) and histamine (100 μM) in the presence of (+)-cicletanine (1 μM) (c) in a group of 6 to 8 HeLa cells. The HeLa cells were exposed to the indicated drugs for a period of 5 mins as indicated by the bars. Typical experiments out of 4 are shown.

Desensitization of the H_1 receptor induced by 100 μM histamine

Desensitization to histamine was observed when the same group of HeLa cells was repeatedly exposed to histamine (Fig. 2 B, C). As depicted in Fig. 2 exposure of cells to 100 μM histamine for 5 mins markedly affected the subsequent $[Ca^{2+}]_i$ transient after a washing period of 5 mins. The intracellular Ca^{2+} levels of the initial peaks for the second and third application of 100 μM histamine were significantly reduced to respectively 590 ± 92 nM (52 % reduction, $n = 6$, $p < 0.05$) and 454 ± 127 nM (65 % reduction, $n = 6$, $p < 0.05$). The second phase was significantly reduced to respecively 279 ± 47 nM (62 % reduction, $n = 6$, $p < 0.05$) and 240 ± 45 nM (70 % reduction, $n = 6$, $p < 0.05$) after 30 secs and further declined to basal Ca^{2+} levels (Fig. 2 B, C).

Thus, an incubation of 5 mins with 100 μM of histamine seems sufficient to induce a rapid loss of responsiveness of the histamine H_1 receptor in HeLa cells. Moreover, histamine-induced desensitization seem to affect two separate components; the release as well as the influx component.

In order to establish whether these observations of receptor desensitization were confined to the histamine H_1 receptor, we examined the effects of histamine pretreatment on the responses to other Ca^{2+}-mobilizing agents. Recent studies performed in our laboratory

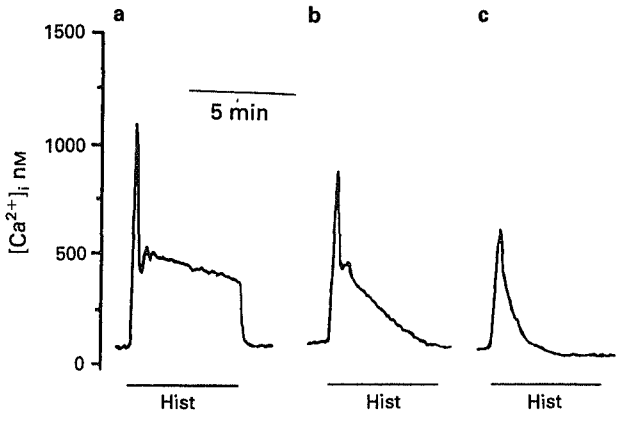

Fig. 2. Desensitization of the histamine-induced Ca^{2+} transients in HeLa cells. The cells were repeatedly exposed to 100 μM of histamine for a period of 5 mins. After the first application of histamine (a) the HeLa cells were washed three times by completely exchanging the medium by rapid perfusion. Histamine was added again after 5 mins (b, c). A typical experiment out of 6 is shown.

showed that in HeLa cells bradykinin, carbachol and ATP can also elevate the $[Ca^{2+}]_i$ levels (data not shown). The responses to bradykinin (1 μM) and carbachol (300 μM) were found to be weak, yet, ATP proved to be effective in elevating $[Ca^{2+}]_i$. Pharmacological investigations revealed that on Hela cells ATP binds to a "5'-nucleotide" type of purinergic receptor, inducing a rapid rise in $[Ca^{2+}]_i$ (Smit *et al.*, 1993). No additivity was monitored when cells were exposed to histamine (100 μM) and ATP (50 μM). Based on these findings, the ATP response was found to be suitable to study whether the desensitization induced by histamine was homologous or heterologous.

In HeLa cells 50 μM ATP-induced a biphasic rise of the $[Ca^{2+}]_i$ levels (Fig. 3A).This response was also characterised by a rapid transient rise in $[Ca^{2+}]_i$, followed by a sustained increase in $[Ca^{2+}]_i$ (Fig. 3A). After application of 50 μM ATP $[Ca^{2+}]_i$ was rapidly elevated from 71 ± 17 nM (n = 6) to 681 ± 140 nM (first phase, n = 6). After this initial rapid rise the $[Ca^{2+}]_i$ decreased to an elevated level of 516 ± 33 nM (second phase, n = 6) after 30 secs of receptor activation.

Pretreatment of HeLa cells with 100 μM histamine for 5 mins did not alter the initial transient response after application of 50 μM ATP (Fig. 3C). After the desensitization with histamine the peak response after stimulation of the ATP receptor was 599 ± 110 nM (n = 4). This value was not significantly different from the peak response under control conditions (681 ± 140 nM, n = 6). However, as for the second and third stimulation with histamine, the second phase of the ATP response was significantly reduced after desensitization with 100 μM histamine (control: 516 ± 33 nM; desensitized: 331 ± 96, n = 4, $p < 0.05$) (Fig. 3C).

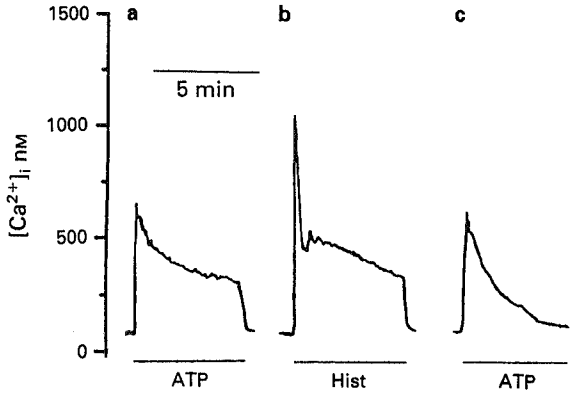

Fig. 3. Intracellular free Ca^{2+} transients induced by ATP (50 µM) in control cells (a) and after (c) desensitization with histamine (Hist, 100 µM) (b) (exposure 5 mins). Experiments were performed as described in Fig. 2. A typical experiment out of 4 is shown.

To investigate the involvement of intracellular Ca^{2+} pools more directly, experiments with caffeine were performed. Caffeine (3 mM) induced a transient rise in $[Ca^{2+}]_i$ in HeLa cells (568 ± 149 nM, n = 4). After the histamine-induced desensitization the response to caffeine did not significantly alter (560 ± 180 nM, n = 4). These data indicate that in contrast to the Ca^{2+} influx the effects of desensitization on the Ca^{2+} release component are confined to H_1 receptor responsiveness.

Role of protein kinase C in the process of desensitization

In many cell systems PKC was found to be responsible for a negative feedback on histamine H_1 receptor mediated responses (Dillon-Carter and Chuang, 1989, Fukui *et al.*, 1991, Leurs *et al.*, 1990, 1991a, Tilly *et al.*, 1990a,b, Volpi *et al.*, 1988). Pretreatment of HeLa cells with increasing concentrations of the protein kinase C (PKC) activator phorbol-12-myristate-13-acetate (PMA), also resulted in a decrease in subsequent responsiveness to histamine (Fig. 4C). Initial responses of histamine were hardly affected by pretreatment of cells with 20 nM PMA for 5 mins. Reasonable reduction was observed following pretreatment with 0.2 µM PMA, whereas complete inhibition was achieved at 2 µM PMA. Based on these observations, a concentration of 0.2 µM of PMA was used for further investigation concerning the role of PKC. Inhibition exerted by PMA on the histamine-induced response resulted particularly in a decrease of the second phase of the Ca^{2+} transient (Fig. 4B). After treatment of HeLa cells with 0.2 µM PMA for 5 mins the $[Ca^{2+}]_i$ levels were only 114 ± 18 nM (n = 6) after 30 secs of stimulation with 100 µM histamine. This value was significantly lower compared to the elevation of the $[Ca^{2+}]_i$ under control conditions (second phase: 601 ± 52 nM). Thus, the

percentage of inhibition by PMA of this second phase of the histamine response is considerably larger compared to the effects on the rapid, initial elevation of $[Ca^{2+}]_i$ (Fig. 4B,C).

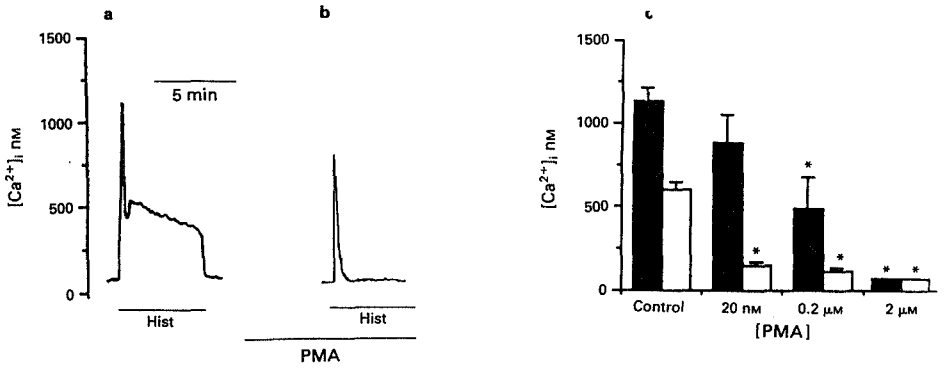

Fig. 4. Intracellular free Ca^{2+} transient induced by histamine (Hist, 100 μM) (a), and by histamine (100 μM) in the presence of 0.2 μM of PMA after 5 mins pretreatment of the cells with 0.2 μM of PMA (b). Effects of increasing concentrations of PMA on the first (closed bars) and second phase (open bars) of the histamine-induced Ca^{2+} response (100 μM) are shown in Fig. 4c. The histamine-induced Ca^{2+} transients were recorded after pretreatment of cells with various doses of PMA for 5 mins, and in the presence of the respective PMA concentration. No histamine-induced Ca^{2+} response was observed when cells were exposed to 2 μM of PMA. Data shown are the mean ± s.e.mean of 6 experiments.

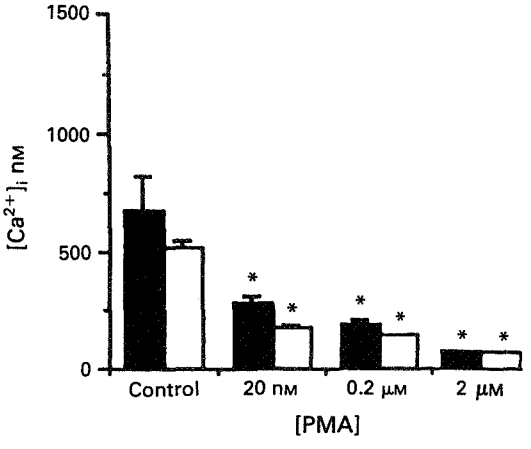

Fig. 5. Effects of increasing concentrations of PMA on the first (closed bars) and second phase (open bars) of the ATP-induced Ca^{2+} response (100 μM). The ATP-induced Ca^{2+} transients were recorded after pretreatment of cells with various doses of PMA for 5 mins, and in the presence of the respective PMA concentration. No ATP-induced Ca^{2+} response was observed when cells were exposed to 2 μM of PMA. Data shown are the mean ± s.e.mean of 6 experiments. The asterisk indicates a significant difference compared to the control histamine-induced response.

To assess the specificity of the inhibitory activity of PMA, we examined the effects of PMA on the ATP response. Pretreatment of HeLa cells with increasing concentrations of PMA resulted in a dose dependent inhibition of the ATP response (50 μM) (Fig. 5).

In order to examine the possible involvement of other regulation mechanisms in the process of desensitization, PKC activity was eliminated by downregulating PKC. This was achieved by incubating HeLa cells for 20 hrs with 0.2 μM PMA, as was done in earlier studies (Tilly *et al.*, 1990a,b). As a control experiment, total exclusion of PKC activity was determined by pretreating the downregulated cells with 0.2 μM PMA for 5 mins (Table 1). Yet, no significant decrease of the responsiveness to histamine was observed after short PMA treatment, justifying the elimination of PKC activity (Table 1).

Table 1 The $[Ca^{2+}]_i$ of the first and second phase of the histamine (100 μM) induced response in control, PKC downregulated and PKC downregulated cells pretreated with 0.2 μM PMA for 5 mins

cell treatment	1st phase $[Ca^{2+}]_i$ (nM)	2nd phase of $[Ca^{2+}]_i$ (nM)	n
control	1135 ± 79	601 ± 52	(11)
PKC downregulated	1121 ± 124	890 ± 90 *	(10)
PKC downregulated + 5 mins. 0.2 μM PMA	1011 ± 156	769 ± 46	(4)

The second phase was recorded 30 secs after receptor activation with histamine. The asterisk indicates a significant difference compared to the control response of histamine. n; number of experiments.

As can be seen in Table 1, histamine still elicited an initial response, which was comparable to the initial response in untreated cells. However, the characteristics of the histamine-induced Ca^{2+} response changed (Fig. 6A). The second phase of the histamine response in these cells significantly ($p < 0.05$) exceeded the Ca^{2+} level of the histamine response in untreated cells. In control cells the $[Ca^{2+}]_i$ levels of the second phase reached 601 ± 52 nM (n = 11), whereas after downregulation of PKC application of histamine resulted in an increase of the second phase to 890 ± 90 nM (n = 6).

When PKC downregulated cells were exposed to another dose of histamine (100 μM), marked desensitization was again observed (Fig. 6B). The initial Ca^{2+} response induced by a second application of histamine was significantly decreased to 519 ± 139 nM (n = 6). Compared to the initial response of the first application (1121 ± 124 nM, Table 1) this corresponded to an inhibition of 54 %. These data imply that homologous desensitization of the histamine H_1 receptor may result from a regulation mechanism other than PKC activation.

Discussion

The histamine H_1 receptor belongs to the class of receptors that is coupled to the phosphatidyl inositol turnover (Haaksma *et al.*, 1990, Hill, 1990). The process of receptor desensitization of this class of receptors remains to be elucidated. In this study we used fluoremetric Ca^{2+}

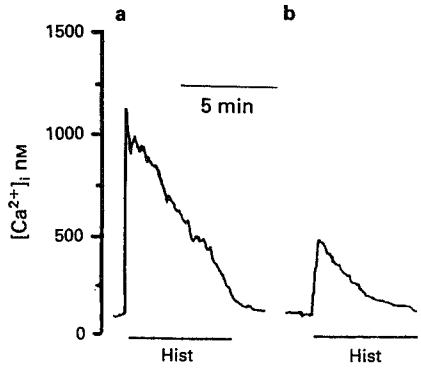

Fig. 6. Histamine-induced Ca^{2+} responses in PKC downregulated cells. The HeLa cells were incubated for 20 hrs with 0.2 μM PMA. After the first application of histamine (Hist, exposure of 5 mins) (a) the cells were washed for 5 mins. and exposed to another dose of histamine (Hist, exposure of 5 mins) (b). A typical experiment out of 6 is shown.

measurements to define the mechanism underlying short-term receptor desensitization of the H_1 receptor in HeLa cells.

Histamine induces an immediate H_1 receptor mediated biphasic rise in $[Ca^{2+}]_i$ in human HeLa cells (Fig. 1). The H_1 receptors on HeLa cells seem to be readily susceptible to desensitization since repetitive exposure of the same group of cells to histamine (100 μM) markedly affected the rise in $[Ca^{2+}]_i$ (Fig. 2). The experimental data show that desensitization of the histamine H_1 receptor is associated with the reduction of both the Ca^{2+} release and influx component. The histamine-induced release component appears to be reduced in a selective manner, as neither the caffeine induced Ca^{2+} release nor the release component of the ATP response were found to be affected. Thus, desensitization of the H_1 receptor does not affect the intracellular Ca^{2+} pool, as was also reported by Hishinuma and Uchida (1988), Leurs *et al.* (1990) and McDonough *et al.* (1988). The influx component, however, of both the histamine and ATP response was found to be impaired. These data indicate that desensitization of the histamine H_1 receptor evolves from two different mechanisms, a selective reduction of the histamine-induced Ca^{2+} release, associated with a homologous desensitization, and a non-selective inhibition of the receptor mediated Ca^{2+} influx, corresponding to a heterologous desensitization.

Receptor desensitization of the GPCRs is thought to be associated with posttranslational modification of the receptor by phosphorylation reactions (Benovic *et al.*, 1988, Hausdorff *et al.*, 1990, Lohse *et al.*, 1990, Sibley *et al.*, 1987). Kinases, such as PKA, PKC and specific receptor kinases seem to play crucial roles in the mechanism underlying receptor desensitization (Huganir and Greengard, 1990, Palczewski and Benovic, 1991). In several receptor systems PKC was found to account for a negative feedback on the histamine H_1 receptor mediated

response (Dillon-Carter *et al.*, 1989, Fukui *et al.*, 1991, Leurs *et al.*, 1990, Tilly *et al.*, 1990a,b, Volpi *et al.*, 1988). This has led to the proposal of a role for PKC in the process of desensitization.

PKC was found to inhibit the histamine as well as ATP responses in HeLa cells, affecting both the Ca^{2+} release and in particular the Ca^{2+} influx (Fig. 4 and 5). A decrease of $^{45}Ca^{2+}$ influx in HeLa cells upon short-term exposure to PMA was also reported by Marunaka *et al.* (1985). Earlier studies showed an impaired formation of inositol phosphates upon H_1 receptor stimulation after activation of PKC in HeLa cells (Tilly *et al.*, 1990a,b). The GTPγS induced formation of inositol phosphates was also found to be reduced, indicating that effects on the G-protein rather than on the H_1 receptor might explain these data (Tilly *et al.*, 1990a). These findings may explain the observed reduction of the initial rise in Ca^{2+} of the histaminergic, as well as purinergic responses after pretreatment with PMA (Berridge and Irvine, 1989). Based upon the model proposed by Irvine, coupling the Ca^{2+} release to the Ca^{2+} influx, the reduced Ca^{2+} release may partly account for the impaired influx of extracellular Ca^{2+} (Irvine, 1990). However, the sensitivity of the Ca^{2+} influx to PMA is much more pronounced, implying that PKC may exert a negative feedback on other molecular entities, such as the receptor operated Ca^{2+} channels. Peppelenbosch *et al.* (1991) developed a model in which the receptor-operated Ca^{2+} influx was suggested to be mediated by transactivation of hyperpolarization-activated Ca^{2+} channels and Ca^{2+}-activated K^+ channels. In this model activation of PKC was proposed to have an inhibitory effect on the K^+ channels leading to a decrease of the Ca^{2+} influx (Peppelenbosch *et al.*, 1991). Since the H_1 receptor activation in HeLa cells is also associated with hyperpolarization of the cell membrane (Hazama *et al.*, 1985, Sauvé *et al.*, 1987, Tilly *et al.*, 1990b), it is likely the H_1 receptor is subjected to a similar negative feedback induced by PKC. This suggestion is further corroborated by the experiments with PKC downregulated HeLa cells (Fig. 6). In these cells the second phase of the histamine-, as well as ATP-induced response was found to be elevated, implying the involvement of PKC in the negative feedback on the influx of extracellular Ca^{2+}.

In contrast to the involvement of PKC in the heterologous desensitization of the Ca^{2+} influx it is highly unlikely that PKC activation is implicated in the selective reduction of the initial H_1 receptor response. Although PKC activation can inhibit the Ca^{2+} release component (see above), this effect is clearly not restricted to the H_1 receptor since also purinergic responses are inhibited. Furthermore, in the PKC downregulated cells, desensitization of the H_1 receptor was still observed (Fig. 6). Based on these data we suggest that during short-term exposure of HeLa cells to histamine besides PKC another regulatory mechanism is rapidly activated. This yet unknown mechanism is responsible for a selective desensitization of the H_1 receptor response. The number of H_1 receptors, measured as [^{3}H]-mepyramine binding sites, did not alter after an incubation of 5 mins with 100 μM of histamine (unpublished observations). Similar findings were monitored in smooth muscle preparations (Leurs *et al.*,

1991b). It is therefore unlikely that short-term desensitization is associated with receptor downregulation. The rapid loss of responsiveness is probably caused by alterations in efficacy of the H_1 receptor to activate the appropriate G-proteins.

During the preparation of this manuscript, the gene encoding the H_1 receptor was cloned by Yamashita *et al.* (1991). Like all other GPCRs, the H_1 receptor was found to contain seven putative transmembrane domains. The β-adrenergic receptor (βAR) is one of the best characterised receptors of the GPCRs. As extensive structural analogy was found between this receptor and the other GPCRs, the βAR provides a model for elucidating characteristics of receptor regulation mechanisms, such as desensitization (Benovic *et al.*, 1988, Hausdorff *et al.*, 1990, Lohse *et al.*, 1990, Sibley *et al.*, 1987). Rapid desensitization of the βAR is associated with phosphorylation of the receptor by protein kinase A (PKA) and the β-adrenoceptor kinase (βARK), resulting in the functional uncoupling of the receptor from its effector system (Benovic *et al.*, 1989). These kinases were found to phosphorylate the receptor at specific serine and threonine residues. The latter kinase is only activated when the receptor is occupied. The primary sequence of the H_1 receptor reveals the existence of several serines and threonines, which may serve as potential phosphorylation sites for such kinases (Yamashita *et al.*, 1991). Based on these findings we therefore hypothesize that the homologous desensitization of the H_1 receptor is associated with the activation of a receptor kinase and the non-specific, heterologous, desensitization with activation of PKC. PKC might play a role comparable to PKA in the process of desensitization. Moreover, up to six βARK related genes have been cloned, implying the existence of a family of receptor kinases, also referred to as G-protein receptor kinases (GRKs) (see for references Lefkowitz, 1993, Haga *et al.*, 1994). These GRKs have distinct substrate specificities (Benovic, 1991). Furthermore, as other GPCRs were found to be substrates for βARK, it is feasible that this class of receptors is regulated by a general mechanism in which specific receptor kinases are responsible for the stimulus-dependent phosphorylation (Benovic *et al.*, 1989). Differences in the number and location of potential phosphorylation sites of various cloned GPCRs, suggest that these receptors might be regulated in distinct ways under different physiological conditions.

In conclusion, results of this study show that histamine induces a rapid H_1 receptor mediated Ca^{2+} response in HeLa cells. PKC appears to be responsible for negative feedback on the Ca^{2+} influx. Moreover, H_1 receptors in HeLa cells are readily susceptible to desensitization. Based upon our experimental data we suggest that short-term desensitisation of the H_1 receptor is mediated by two distinct pathways, a PKC dependent and PKC independent pathway. In view of future experiments, the recent cloning of the histamine H_1 receptor (Yamashita *et al.*, 1991) offers new possibilities, with regard to the elucidation of the mechanism underlying receptor desensitization.

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Chapter 3

Regulation of the human histamine H_1 receptor stably expressed in Chinese Hamster Ovary cells

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In the present study we characterized the human H_1 receptor expressed in Chinese Hamster Ovary cells ($CHOhumH_1$ cells) and examined whether this receptor was susceptible to short-term and long-term receptor desensitization. The human H_1 receptor gene expressed in Chinese Hamster Ovary cells encodes a classical histamine H_1 receptor with a pharmacology similar to that of the H_1 receptor found in guinea-pig cerebellum and the endogenously expressed human H_1 receptor in 1321N1 astrocytoma cells. Histamine induced a dose-dependent rise in inositol phosphates (EC_{50}: 2.2 ± 1.0 μM) and a rapid increase of $[Ca^{2+}]_i$, followed by a sustained increase of $[Ca^{2+}]_i$ upon addition of 100 μM histamine.

Short-term exposure of the human H_1 receptor to histamine led to an heterologous desensitization of the agonist-induced Ca^{2+} response, which may be explained by alterations at the level of the intracellular Ca^{2+} pool. In $CHOhumH_1$ cells the PKC activator, PMA (1 μM), was found to inhibit the histamine (100 μM)- as well as the ATP (100 μM)-induced Ca^{2+} reponse only partially and less effective than histamine-pretreatment. Moreover, in $CHOhumH_1$ cells PKC downregulation induced by long-term exposure to PMA (1 μM) did not affect the histamine-induced desensitization nor did pretreatment with the PKC inhibitor staurosporine (100 nM), indicating that in $CHOhumH_1$ cells PKC is probably not involved in the heterologous desensitization.

Long-term treatment of $CHOhumH_1$ cells with histamine or other H_1 agonists resulted in a time- and dose-dependent decrease in the number of H_1 receptor binding sites (maximal reduction: 47 ± 5%). Long-term exposure of $CHOhumH_1$ cells to ATP or PKC did not affect H_1 receptor density, indicating that H_1 receptor downregulation is apparently not induced by activation of phospholipase C or PKC. Both histamine (100 μM)- and ATP (100 μM)-induced Ca^{2+} responses were affected upon long-term exposure of cells to histamine (100 μM), which might be explained by an alteration at a level distant from the receptor.

Taken together, these findings show that in $CHOhumH_1$ cells the human histamine H_1 receptor is susceptible to short-term and long-term receptor regulation in which PKC does not seem to play a role. The $CHOhumH_1$ cells therefore provide an excellent model system to study the mechanism(s) of PKC-independent H_1 receptor regulation.

Introduction

The gene encoding the histamine H_1 receptor was successfully cloned from bovine adrenal medulla by means of an expression cloning strategy (Yamashita *et al.*, 1991). Soon thereafter, the guinea-pig (Horio *et al.*, 1993, Traiffort *et al.*, 1994), rat (Fujimoto *et al.*, 1993) and human homologues (De Backer *et al.*, 1993, Fukui *et al.*, 1994, Moguilevsky *et al.*, 1994) were cloned by homology screening. The cloning of these genes revealed that the histamine H_1 receptor belongs to the multigene family of G-protein coupled receptors, which putatively all contain seven hydrophobic transmembrane domains, separated by hydrophilic intra- and extracellular loops (Collins, 1993).

For several members of the family of G-protein coupled receptors it has been shown that excessive stimulation of the receptor protein leads to a modulation of the receptor response and regulation of receptor expression (Collins, 1993, Lohse, 1993). Short-term activation of the histamine H_1 receptor is indeed frequently followed by a period of refractoriness, often

referred to as desensitization. H_1 receptor desensitization has been observed and studied in several isolated tissues (Bristow *et al.*, 1993, Dillon-Carter and Chuang, 1989, Leurs *et al.*, 1990, Quach *et al.*, 1981) and cultured cell lines (Bristow and Zamani, 1993, Brown *et al.*, 1986, Dickenson and Hill, 1993, McCreath *et al.*, 1994, McDonough *et al.*, 1988, Smit *et al.*, 1992, Zamani *et al.*, 1994). Moreover, desensitization of H_1 receptor responses has also been observed *in vivo* (Antol *et al.*, 1988, Manning *et al.*, 1987, Poulakos and Gertner, 1986), indicating the importance of this process in physiology. So far no clear mechanistic details are known, although, depending on the cell type, protein kinase C (PKC)-dependent and independent mechanisms for short-term H_1 receptor desensitization have been observed (Cowlen *et al.*, 1990, Dickenson and Hill, 1993, Dillon-Carter and Chuang, 1989, Fukui *et al.*, 1991, Leurs *et al.*, 1990, McCreath *et al.*, 1994, Smit *et al.*, 1992). Similarly, both receptor specific (homologous)- and receptor non-specific (heterologous) desensitization have been observed in various cellular systems (Brown *et al.*, 1986, Cowlen *et al.*, 1990, Dickenson and Hill, 1993, Dillon-Carter and Chuang, 1989, Leurs *et al.*, 1990, McCreath *et al.*, 1994, McDonough *et al.*, 1988, Quach *et al.*, 1981, Smit *et al.*, 1992). Homologous desensitization is usually considered to be due to a specific modification of the receptor protein, whereas heterologous desensitization is often caused by a modulation of a common signal transduction pathway.

Prolonged exposure of the G-protein coupled receptors to their respective agonist often results in a reduction in receptor number (Collins, 1993, Lohse, 1993). For the H_1 receptor only one experimental study has been published, reporting a 20% downregulation of the H_1receptor after an *in vitro* histamine treatment of mouse brain tissue (Quach *et al.*, 1981). Yet, there are various reports that show that the H_1 receptor density is dynamically regulated *in vivo*. Yanai *et al.* (1992) showed by means of [^{11}C]-doxepin positron emmission tomography (PET) studies that the H_1 receptor density in the brain decreases upon aging. Using the same technique Iinuma *et al.* (1993) revealed an upregulation of H_1 receptors in the electrical focii in the temporal cortex of epileptic patients. Moreover, in the frontal cortex of patients with chronic schizophrenia a downregulation of H_1 receptors was reported (Nakai *et al.*, 1991). These examples indicate that the H_1 receptor expression is susceptible to regulatory changes under physiological and pathophysiological conditions, emphazising the need to further delineate the mechanisms underlying the process of H_1 receptor regulation.

The recent cloning of the gene encoding the histamine H_1 receptor permits a more detailed investigation of the molecular mechanisms, related to the H_1 receptor function. Especially investigations of the downregulation of the H_1 receptor have been hampered so far by the lack of suitable model systems with a reasonable density of H_1 receptors. We therefore stably transfected the gene encoding the human H_1 receptor into Chinese Hamster Ovary (CHO) cells. First, the expressed human H_1 receptor was subjected to a pharmacological characterization. Thereafter this cell line was used to investigate whether the expression of the

human H_1 receptor can be modulated by prolonged exposure to histamine. Moreover, we also studied the effects short-term exposure to histamine to evaluate the use of transfected CHO cells in mechanistic studies on homologous H_1 receptor desensitization. In all these studies the role of protein kinase C in the regulatory processes was evaluated.

Materials and methods

Transfection and cell culture Chinese Hamster Ovary (CHO) cells, deficient in dihydrofolate reductase, were maintained at 37°C in a humidified incubator with 95% air and 5% CO_2 in α-minimal essential medium (αMEM) with ribonucleosides and deoxyribonucleosides supplemented with L-glutamine (2mM), penicillin (50 IU/ml), streptomycin (50 µg/ml) and 10% (vol/vol) foetal calf serum. CHO cells were stably transfected with the eukaryotic expression vector pdKCR-dhfr$^-$ containing the human H_1 receptor gene using the calcium phosphate precipitation method as described previously by Fujimoto *et al.* (1993). Selection was imposed by growing the cells in α-MEM without ribonucleosides and deoxyribonucleosides supplemented with L-glutamine (2mM), penicillin (50 IU/ml), streptomycin (50 µg/ml) and 10% (vol/vol) dialyzed foetal calf serum, which resulted in the appearance of several clonal cell lines. Thereafter, these cells were screened for expression of [^{3}H]-mepyramine binding.

CHO cells stably expressing the human H_1 receptor were cotransfected with the PKC isoenzymes α, β_I, β_{II}, δ, ε or γ cDNAs, inserted into a pTB vector (Ono *et al.*, 1987), and the pSV$_2$neo vector using the calcium phosphate method as described previously by Fujimoto *et al.* (1993). Neomycin resistant clones were selected by growing the cells in medium containing 500 µg/ml of G418. The clones were screened by means of [^{3}H]-phorbol-12,13-dibutyrate binding.

The human 1321N1 human astrocytoma cells (Nakahata *et al.*, 1985) were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbeco's Modified Eagle's Medium (DMEM), containing 10% foetal calf serum and supplemented with 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin.

Membrane preparation CHOhumH_1 cells and 1321N1 cells were harvested by means of a cell scraper and recovered by a 10 min centrifugation at 500g. Cells were homogenized in ice-cold 50 mM Na_2/K-phosphate buffer (pH = 7.4) with a Polytron homogenizer (5 secs, maximal speed) and used for radioligand binding studies. Protein concentrations were determined according to Bradford (1976) (Bradford, 1976) using bovine serum albumin as a standard.

Histamine H_1 receptor binding Membranes (60-80 µg of protein) were incubated for 30 min at 25 °C in 50 mM Na_2/K phosphate buffer in a total volume of 400 µl with the indicated concentrations of [^{3}H]-mepyramine. In saturation studies increasing concentrations of [^{3}H]-mepyramine were incubated with the membranes in the absence or presence of 1 µM mianserin. In displacement studies membranes were incubated with 2 nM [^{3}H]-mepyramine and increasing concentrations of competing ligands. The incubations were stopped by rapid dilution with 3 ml ice-cold 50 mM Na_2/K phosphate buffer (pH = 7.4). The bound radioactivity was subsequently separated by filtration with a Brandel cell harvester (Semat, UK) through Whatman GF/B glass fibre filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 ml buffer and radioactivity retained on the filters was measured by liquid scintillation counting. The binding data were evaluated using LIGAND, a non-linear, weighted, least squares curve-fitting procedure (Munson and Rodbard, 1980).

Changes in H_1 receptor density were denoted as a percentage downregulation compared to non-treated control cells. During the 24 hrs incubation of cells with various histaminergic ligands or other compounds, cells were grown in serum-free medium.

[^{3}H]-Inositol phosphate measurements CHOhumH_1 cells were seeded in 12-well plates and cultured overnight in culture medium. Thereafter, cells were labeled overnight in inositol-free medium supplemented with 1 µCi/ml [^{3}H]-*myo*-inositol. Cells were washed twice with Krebs buffer (125 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2.2H_2O$, 1.2 mM $MgCl_2.6\ H_2O$, 1.2 mM $KH_2PO_4.2H_2O$, 11 mM glucose, 15 mM Hepes, 15 mM $NaHCO_3$, pH = 7.4 at 37°C), supplememted with 10 mM LiCl, and preincubated for 10 min at 37 °C with or without one of the histaminergic antagonists at the indicated concentrations. Incubations were started by the addition of histamine. After 10 min incubation at 37 °C the medium was aspired and the reaction was stopped by addition of 5% TCA. The cells were chilled on ice for 10 mins. Thereafter, 2 ml of water-saturated diethylether was added to the TCA extract and was mixed for 5 mins. The ether phase was aspired and the procedure was repeated once more. After aspiration the remaining diethylether was removed by incubation at 37°C. 150 µl 0.2

M Tris-HCl was added to neutralize the samples and the [^{3}H]-inositol phosphates were isolated by anion exchange chromatography (Godfrey, 1992).

Measurements of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) CHOhumH$_1$ cells were trypsinized, washed with αMEM supplemented with 0.2% bovine serum albumin (BSA) and resuspended in αMEM/ 0.2%BSA containing 2 μM fura-2-AM and 0.025% Pluronic-F for 1 hr at 25 °C. Thereafter cells were gently washed with 5 ml of αMEM/0.2% BSA, resuspended in 1 ml of αMEM/0.2% BSA and stored on ice. A 100 μl sample was added to 1.5 ml prewarmed Tyrode solution (2.5 mM KCl, 0.5 mM $MgCl_2$, 137 mM NaCl, 0.36 mM NaH_2PO_4, 5.6 mM glucose and 10 mM Hepes, pH = 7.4) in a temperature-controlled (30°C) quartz cuvette. Ca^{2+}-dependent fura-2 fluorescence was monitored at an emission wavelength of 510 nm with an excitation wavelength of 340 and 380 nm in a Shimadzu RF5001PC. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to the formula derived by Grynkiewicz (1985). Maximal fluorescence was measured (F_{max}) by permeabilization of cells with 0.2% Triton-X-100 and minimal fluorescence (F_{min}) was achieved by complexing calcium with 10 mM EGTA.

Desensitization experiments were carried out with CHOhumH$_1$ cells grown on a coverslip. Cells were loaded with 2 μM fura-2-AM in a HBS buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 0.2% BSA, 10 mM Hepes, pH = 7.4) for 1 hr at 25 °C. Thereafter, the coverslip was inserted into the quartz cuvette and cells were washed by completely exchanging prewarmed HBS buffer (30°C). Fluorescence was measured as described above.

[^{3}H]-phorbol-12,13-dibutyrate binding CHOhumH$_1$ cells were seeded in 12-well plates and cultured overnight in culture medium. Cells were washed with PBS and preincubated for 10 min in αMEM supplemented with 0.1% BSA and 20 mM Hepes (pH 7.0) (binding buffer) at 37 °C. Thereafter, cells were incubated in binding buffer with 5 nM [^{3}H]-phorbol-12,13-dibutyrate in the presence or absence of 10 μM PMA for 1 hr at 37 °C. The incubations were stopped by washing with ice-cold PBS buffer supplemented with 0.1% BSA. Next, cells were incubated with trypsine-EDTA for several hours in order to disrupt the cells. The bound radioactivity was subsequently separated by filtration through GF/B glass fibre filters. Filters were washed with cold PBS and the radioactivity retained on the filters was counted. The difference in radioactivity measured in cells incubated in the absence and presence of 10 μM PMA was considered as specific phorbolester binding.

Chemicals Histamine dihydrochloride, phorbol-12-myristate-13-acetate, phorbol-12,13-dibutyrate (PdBu), 4α-phorbol, bovine serum albumin (BSA), caffeine and ATP (disodium salt) were obtained from Sigma Chemical Company (USA). Dowex AG1x8 (200-400 mesh) formate form was purchased from Bio-rad (Bio-rad laboratories GmbH, Germany). [^{3}H]-mepyramine ([pyridinyl-5-^{3}H]-pyrilamine, 21 Ci/mmol), [^{3}H]-phorbol-12,13-dibutyrate (15.1 Ci/mmol) and [^{3}H]-*myo*-inositol (18.8 Ci/mmol) were obtained from Amersham. Fura-2-acetoxymethylester (fura-2-AM) and Pluronic-F was purchased from Molecular Probes (Eugene, OR, USA). 2-pyridylethylamine dihydrochloride was taken from laboratory stock. Gifts of mianserin (Organon, The Netherlands), 2-methylhistamine dihydrochloride, 2-thiazolylethylamine dihydrochloride (SmithKline Beecham, United Kingdom), the enantiomers of cicletanine (Institute of Henri Beaufour, France), the enantiomers of chlorpheniramine (maleate salts) (Dr. A. Belt, Nijmegen, The Netherlands) and the PKC isoenzyme cDNAs (Drs. Kikkawa and Ono, Kobé University, Japan) are greatly acknowledged.

Statistical analysis All data shown are expressed as mean ± standard errors (mean ± s.e.mean) of at least three independent experiments. Statistical analysis was carried out by Student's *t*-test. P-values < 0.05 were considered to indicate a significant difference.

Results

Pharmacological characterization of the human histamine H_1 receptor expressed in Chinese Hamster Ovary cells (CHOhumH$_1$)

Transfection of the cDNA encoding the human H$_1$ receptor (Fukui *et al.*, 1994) into CHO cells resulted in the isolation of several clones, expressing [^{3}H]-mepyramine binding sites. Untransfected cells did not show any specific [^{3}H]-mepyramine binding. One clonal cell line (CHOhumH$_1$) was selected for further analysis. Saturation experiments performed with CHOhumH$_1$ cell membranes revealed the presence of a single population of saturable [^{3}H]-

mepyramine binding sites with a dissociation constant (K_d) of 1.10 ± 0.09 nM and a maximal density (B_{max}) of 861 ± 41 fmol/mg protein (n = 3, mean ± s.e.mean) (Fig. 1A). The binding of 2 nM [^{3}H]-mepyramine to CHOhumH$_1$ cell membranes was monophasically and stereoselectively inhibited by various H_1 antagonists (Fig. 1B, Table 1). For comparison, binding studies were performed on membranes of human 1321N1 astrocytoma cells, which endogenously express a low level of H_1 receptors (Nakahata *et al.*, 1985). Saturation experiments revealed a K_d value of [^{3}H]-mepyramine of 1.14 ± 0.22 nM and a B_{max} of 131 ± 46 fmol/mg protein (n = 3, mean ± s.e.mean). The pK_i values for the different H_1 antagonists on CHOhumH$_1$ membranes were found to be consistent with the observed pK_i values on 1321N1 membranes and reported pK_i values determined on guinea-pig cerebellum (Ter Laak *et al.*, 1993) (Table 1). Histamine displaced the specific [^{3}H]-mepyramine binding to CHOhumH$_1$ membranes with a pK_i value of 4.67 ± 0.02 (n = 5, mean ± s.e.mean), a value that closely corresponds to the pK_i-value previously determined with 1321N1 astrocytoma membranes (Nakahata *et al.*, 1985).

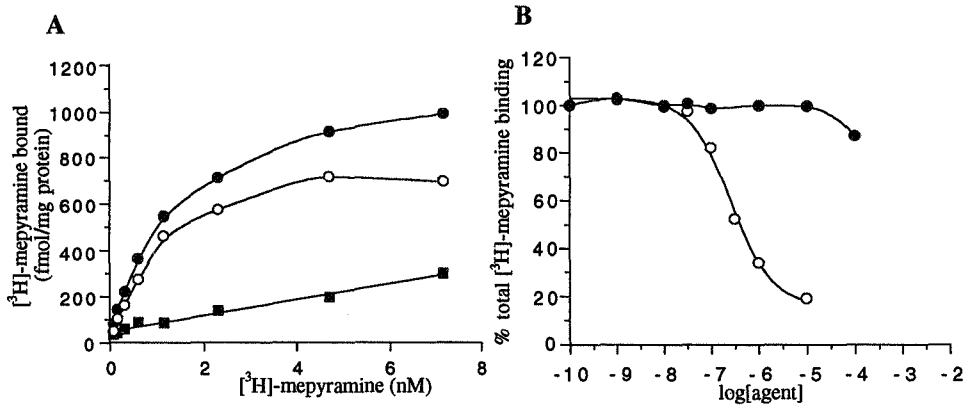

Fig. 1. Binding of [^{3}H]-mepyramine to CHOhumH$_1$ cell membranes. A, Saturation of [^{3}H]-mepyramine to CHOhumH$_1$ cell membranes. Specific radioligand binding (open circles) was determined by substracting the binding determined in the presence of 1 μM of mianserin (filled squares) from the total binding (filled circles). B, Displacement of binding of 2 nM [^{3}H]-mepyramine by increasing concentrations of (-)-cicletanine (open circles) and (+)-cicletanine (filled circles). Mean ± the s.e.mean values of triplicate determinations of a typical experiment out of three are shown.

Experiments with [^{3}H]-*myo*-inositol labelled CHOhumH$_1$ cells showed a dose-dependent increase in inositol phosphate production when cells were incubated for 10 min with increasing concentrations of histamine (Fig. 2A). The EC_{50} value of histamine for this response was 2.2 ± 1.0 μM (n = 3, mean ± s.e.mean). A 10-fold stimulation over basal levels of inositol phosphate production was observed when cells were stimulated with 100 μM histamine. The H_1 antagonist mepyramine (1 μM) inhibited the histamine (100 μM)-induced production of inositol phosphates, whereas the H_2 antagonist ranitidine (10 μM) and the H_3 antagonist

thioperamide (1 μM) did not affect the histamine-induced inositol phosphate response (Fig. 2A inset).

Table 1 Characterization of [^{3}H]-mepyramine binding to CHOhumH$_1$ cell membranes

	pK$_i$		
antagonists	guinea-pig cerebellum	1321N1 cells	CHOhumH$_1$ cells
(+)-cicletanine	< 5	< 5	< 5
(-)-cicletanine	7.54	7.74 ± 0.04	7.27 ± 0.06
(d)-chlorpheniramine	8.32	8.35 ± 0.20	8.45 ± 0.04
(l)-chlorpheniramine	6.68	6.78 ± 0.12	6.53 ± 0.06

Membranes of CHOhumH$_1$ cells and 1321N1cells were incubated with 2 nM [^{3}H]mepyramine in the presence of the indicated drugs at increasing concentrations. K$_i$ values were obtained from the respective IC$_{50}$ values. Data shown are the mean ± s.e.mean from three independent experiments. Reported values for [^{3}H]-mepyramine binding to guinea pig cerebellum (Ter Laak *et al.*, 1993) are shown for comparison.

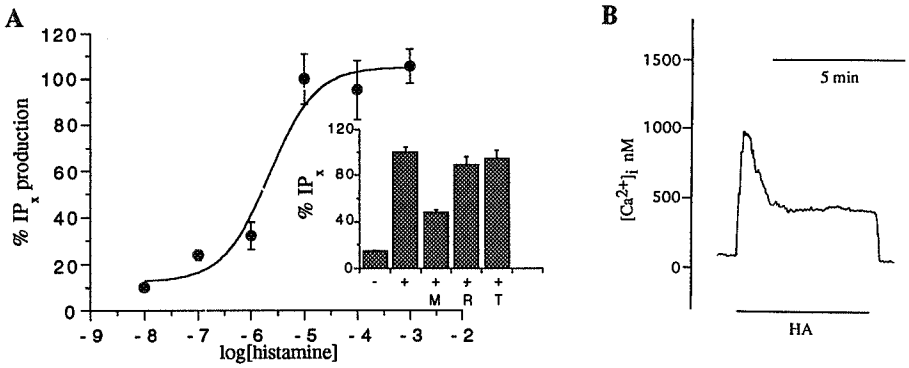

Fig. 2. Signal transduction pathways of the human H$_1$ receptor expressed in CHO cells. A, Histamine-induced production of [^{3}H]-inositol phosphates. Cells were prelabeled overnight with [^{3}H]-*myo*-inositol, were washed twice and preincubated for 10 mins in αMEM containing 10 mM LiCl. Thereafter, cells were incubated for 10 mins with increasing concentrations of histamine in the same medium. (basal inositol phosphate levels: 2127 ± 371 dpm/well, histamine induced increases of inositol phosphate production: 23449 ± 2872 dpm/well, n = 3, mean ± s.e.mean). Mean ± the s.e. values of triplicate determinations of a typical experiment out of three are shown. (Inset) Effect of the histamine H$_1$ antagonist mepyramine (M) (1 μM), H$_2$ antagonist ranitidine (R) (10 μM) and H$_3$ antagonist thioperamide (T) (1 μM) on the histamine-induced increase in inositol phosphate production over basal levels (-) (B) (n = 2). Cells were preincubated for 10 mins with the antagonists. Thereafter, cells were stimulated with 100 μM histamine (+). B, Histamine-induced increase in [Ca^{2+}]$_i$ in fura-2/AM-loaded CHOhumH$_1$ cells grown on a coverslip. Cells were incubated at 30°C in HBS buffer and exposed to 100 μM histamine for 5 mins and washed with HBS buffer. Data shown are from representative experiment (basal [Ca^{2+}]$_i$: 91 ± 4 nM, histamine-induced increase [Ca^{2+}]$_i$: 1040 ± 53 nM, n = 8, mean ± s.e.mean).

Addition of 100 μM histamine to fura-2-loaded CHOhumH$_1$ cells resulted in a rapid increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (basal $[Ca^{2+}]_i$: 91.4 ± 3.8 nM, histamine-induced increase $[Ca^{2+}]_i$: 1040 ± 53 nM, n = 8, mean ± s.e.mean) (Fig. 2B). The rapid transient increase was followed by a sustained increase in $[Ca^{2+}]_i$ (477 ± 27 nM) lasting until the agonist was removed. Preincubation of these cells with 1 μM of the H$_1$ antagonist mepyramine for a period of 10 min prior to stimulation with histamine (100 μM), totally blocked the histamine-induced increase of $[Ca^{2+}]_i$ (data not shown).

Short-term desensitization of the human histamine H_1 receptor expressed in CHO cells

Desensitization of the histamine-induced Ca^{2+} response in CHOhumH$_1$ cells was observed when the same cells were repeatedly exposed to histamine (Fig. 3). After the first challenge with 100 μM histamine for a period of 5 min, cells were washed for 5 mins and re-exposed to 100 μM histamine for another 5 min. Thereafter, this procedure was repeated once more. In total therefore 3 histamine-induced Ca^{2+} responses were recorded. As depicted in Fig. 3 the consecutive responses we markedly affected by the previous challenges to histamine (basal $[Ca^{2+}]_i$: 91 ± 4 nM, first histamine-induced increase $[Ca^{2+}]_i$: 1040 ± 53 nM, n = 8, second histamine-induced increase $[Ca^{2+}]_i$: 458 ± 60 nM (reduction 56 ± 6%, n = 6), third histamine-induced increase $[Ca^{2+}]_i$: 169 ± 12 nM (reduction of 84 ± 1%, n = 6, mean ± s.e.mean).

In CHOhumH$_1$ cells 100 μM ATP also induced a rapid rise of the $[Ca^{2+}]_i$, followed by a sustained increase in $[Ca^{2+}]_i$ (basal $[Ca^{2+}]_i$ 78.5 ± 10.5 nM, ATP-induced increase $[Ca^{2+}]_i$: 1489 ± 131 nM, n = 3, mean ± s.e.mean). Pretreatment of these CHOhumH$_1$ cells with 100 μM histamine for 5 mins affected the Ca^{2+} response induced by 100 μM ATP too. A decrease

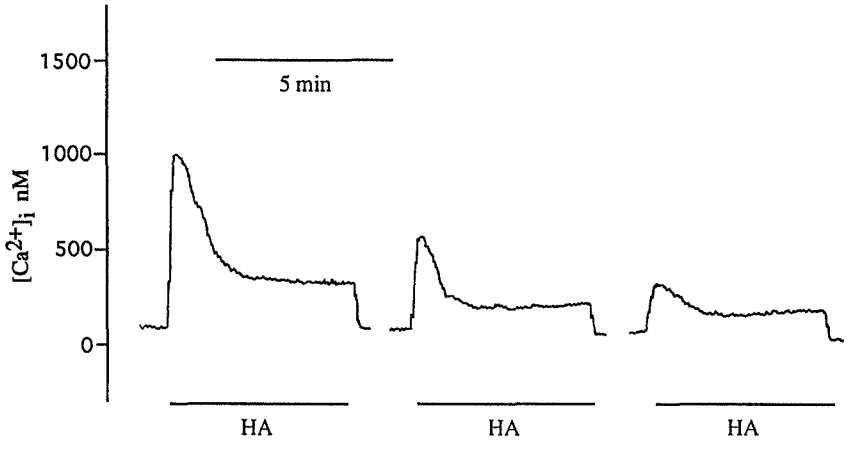

Fig. 3. Desensitization of the histamine-induced Ca^{2+} transients in CHOhumH$_1$ cells. Cells were repeatedly exposed to 100 μM histamine (HA) for a period of 5 mins. After each histamine challenge cells were washed for 5 mins by completely exchanging the buffer. A typical experiment out of 6 is shown.

of 63 ± 5% (n = 4, mean ± s.e.mean) was observed when CHOHuH$_1$ cells were pretreated for 5 mins with histamine. Preincubation of cells with 100 μM ATP for 5 mins resulted also in a 66 ± 3% (mean ± s.e.mean, n = 3) attenuation of the histamine-induced Ca^{2+} response. Caffeine (10 mM) induced a rise in $[Ca^{2+}]_i$ in CHOhumH$_1$ cells (332 ± 23 nM, n = 4, , mean ± s.e.mean). After a 5 mins pretreatment of cells with 100 μM histamine, the caffeine response was significantly reduced to 45 ± 5% of the control response (n = 3, mean ± s.e.mean).

Role of protein kinase C in the process of short-term desensitization

Pretreatment of CHOhumH$_1$ cells for 10 mins with 1 μM of the PKC activator PMA, a concentration shown to be maximally effective in CHO cells (Leurs *et al.*, 1994) resulted in a decrease of 46 ± 7% (mean ± s.e.mean, n = 3) of the histamine-induced Ca^{2+} response, whereas pretreatment with the inactive phorbol ester 4α-phorbol did not affect the histamine-induced Ca^{2+} response. The ATP-induced Ca^{2+} response was also found to be inhibited for 40 ± 2% (mean ± s.e.mean, n = 3) upon 1 μM PMA pretreatment.

PKC downregulation by 18 hrs incubation of cells with 1 μM PMA did, however, not affect the histamine-induced desensitization (control cells: 56 ± 4% desensitization, PKC downregulated cells: 66 ± 9% desensitization, n = 6, mean ± s.e.mean). Moreover, exposure of cells to the PKC inhibitor staurosporine (100 nM) for 30 mins prior to and during histamine pretreatment did not inhibit the histamine-induced desensitization of the histamine-induced Ca^{2+} reponse (control cells: 56 ± 4% desensitization, n = 6, staurosporine-treated cells: 73 ± 5% desensitization, n = 4, mean ± s.e.mean, $p < 0.05$).

Long-term desensitization of the human histamine H_1 receptor expressed in CHO cells

Incubation of CHOhumH$_1$ cells with 100 μM histamine for periods ranging from 2 to 32 hrs resulted in a time dependent decrease of [^{3}H]-mepyramine binding (Fig. 4 A). A maximal reduction (n = 3, mean ± s.e.mean) was observed after 24 hrs incubation of cells with 100 μM histamine. Half-maximal decrease of [^{3}H]-mepyramine binding was recorded after an incubation period of about 14 hrs. Saturation studies showed that the K_d value of [^{3}H]-mepyramine was not affected by 24 hrs incubation of cells with 100 μM histamine (Table 2). Long-term exposure (24 hrs) of CHOhumH$_1$ cells with 100 μM histamine resulted in a reduction (47 ± 5%, n = 3, mean ± s.e.mean) of the total number of [^{3}H]-mepyramine binding sites (B_{max}) (Table 2). The decrease of [^{3}H]-mepyramine binding was shown to be dose-dependent, as 24 hrs incubation of CHOhumH$_1$ cells with increasing concentrations of histamine resulted in a dose-dependent reduction of [^{3}H]-mepyramine binding, with an EC_{50} of 0.13 ± 0.02 μM (n = 3, mean ± s.e.mean) (Fig. 4B). Exposure of CHOhumH$_1$ cells for 24 hrs with H$_1$ agonists (2-pyridylethylhistamine (2-PEA), 2-methylhistamine (2-MeHA) and 2-thiazolylethylamine (2-TEA)) also reduced the [^{3}H]-mepyramine binding (inset Fig. 4B).

Table 2 Characteristics of the [^{3}H]-mepyramine binding to the human histamine H_1 receptor expressed in CHO cells pretreated for 24 hrs with or without histamine

pretreatment 24 hrs	K_d (nM)	B_{max} (fmol/mg protein)
control	1.10 ± 0.09	861 ± 41
histamine (100 μM)	0.98 ± 0.10	460 ± 46 *

The dissociation constant (K_d) and maximal number of binding sites (B_{max}) were determined using non-linear regression analysis according to a one-site binding mode. The data shown represent the mean ± s.e.mean of 3 independent experiments. The asterisk indicates a significant difference ($p < 0.05$) from control, represented by untreated cells.

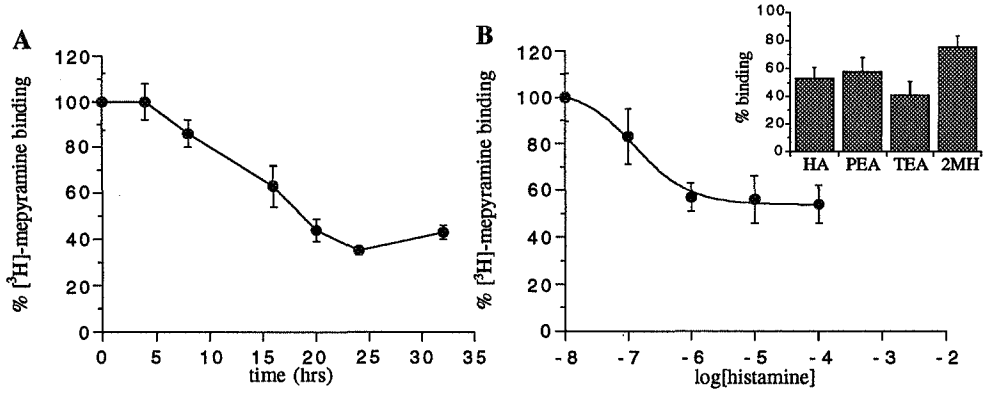

Fig. 4. Histamine-induced reduction of [^{3}H]-mepyramine binding in CHOhumH$_1$ cells. A, CHOhumH$_1$ cells were incubated with 100 μM histamine for the indicated times and [^{3}H]-mepyramine binding in membranes was measured. The [^{3}H]-mepyramine binding is expressed as a percentage of [^{3}H]-mepyramine binding measured in non-treated cells. The data shown represent the mean ± s.e.mean of 3 independent experiments. B, Dose-dependent decrease of [^{3}H]-mepyramine binding induced by histamine. CHOhumH$_1$ cells were exposed to various concentrations of histamine for 24 hrs. The data represent the mean ± s.e.mean of 3 independent experiments. (Inset) Effect of pretreatment with H_1 agonists on [^{3}H]-mepyramine binding of CHOhumH$_1$ cells. CHOhumH$_1$ cells were incubated for 24 hrs with histamine (HA, 100 μM), 2-PEA (PEA, 100 μM), 2-TEA (TEA, 100 μM) and 2-MeHA (2MH, 100 μM). [^{3}H]-mepyramine binding to the membranes was measured as described above. Data were calculated as the mean ± s.e.mean from at least 3 independent experiments.

Role of PKC in the process of histamine-induced H_1 receptor downregulation

Exposure of CHOhumH$_1$ cells for 24 hrs to 0.1 μM or 1 μM PMA did not affect [^{3}H]-mepyramine binding (Table 3). Incubations of CHOhumH$_1$ cells with 1 μM PMA for shorter periods of time (4 hrs) also did not affect [^{3}H]-mepyramine binding (data not shown). Similar observations were found for incubation of cells with 1 μM PdBu. Concurrent exposure of CHOhumH$_1$ cells to 100 μM histamine and 1 μM PMA for 24 hrs caused a significant reduction of [^{3}H]-mepyramine binding, which was less pronounced than treatment with histamine alone (Table 3). Co-incubation of cells with PMA (1 μM) and histamine (100 μM) for 4 hrs, did not result in reduction of [^{3}H]-mepyramine binding (data not shown). Exposure

Table 3 Effect of long-term PKC and P_{2U}-receptor activition on [^{3}H]-mepyramine binding of CHOhumH_1 cells

compounds		% downregulation	n
histamine	100 μM	41 ± 5 *	11
PMA	0.1 μM	-13 ± 5	6
PMA	1 μM	-5 ± 5	18
PDBu	1 μM	-22 ± 10	4
histamine (100 μM)+ PMA (1 μM)		27 ± 3 *	5
ATP	100 μM	15 ± 8	6

CHOhumH_1 cells were incubated for 24 hrs with the indicated compounds and [^{3}H]-mepyramine binding to the membranes was measured. The [^{3}H]-mepyramine binding is expressed as a percentage of downregulation compared to non-treated cells. Data were calculated as the mean ± s.e.mean from n independent experiments. The asterisk indicates a significant difference ($p < 0.05$) from control, represented by untreated cells.

of CHOhumH_1 cells to 100 μM ATP for 24 hrs did also not induce a change in the number of [^{3}H]-mepyramine binding sites (Table 3).

Effect of long-term desensitization on histamine- and ATP-induced Ca^{2+} signalling

A marked attenuation of the 100 μM histamine-induced Ca^{2+} response was observed when the CHOhumH_1 cells were previously exposed to 100 μM histamine for 24 hrs (reduction of 57 ± 4%, n = 4, mean ± s.e.mean). This treatment also impaired the ATP-induced rise (100 μM) in Ca^{2+} (reduction 32 ± 6%, n = 4, mean ± s.e.mean).

Incubation of CHOhumH_1 cells that had been exposed to 100 μM histamine for 24 hrs, for 10 mins with 1 μM PMA resulted in a further attenuation of the 100 μM histamine- and 100 μM ATP-induced Ca^{2+} response (45 ± 4% and 43 ± 6%, respectively, n = 3, mean ± s.e.mean).

Discussion

In the present study we have shown that the human H_1 receptor cDNA expressed in CHO cells encodes a classical histamine H_1 receptor with a pharmacology similar to that of the H_1 receptor found in guinea-pig cerebellum (Ter Laak *et al.*, 1993) and the endogenously expressed human H_1 receptor in 1321N1 astrocytoma cells (Nakahata *et al.*, 1985, present study) (Table 1). The pK_i values of different H_1 receptor antagonists, including their respective enantiomers, are in accordance with the reported pK_i values determined on guinea-pig cerebellum and values found for 1321N1 astrocytoma cells (Table 1). Similar results were recently reported for the human H_1 receptors, expressed into respectively COS-7 and CHO cells (De Backer *et al.*, 1993, Moguilevsky *et al.*, 1994). Although a few studies on H_1 receptor signalling of the cloned guinea-pig (Leurs *et al.*, 1994) and bovine (Iredale *et al.*, 1993) H_1 receptor, both expressed in CHO cells, have been reported our study is the first describing H_1 receptor signalling after stable transfection of the human H_1 receptor gene. In

CHO cells stably expressing the human H_1 receptor (CHOhumH_1), histamine induced a dose-dependent rise in inositol phosphates and a rapid increase of $[Ca^{2+}]_i$, followed by a sustained increase of $[Ca^{2+}]_i$ upon addition of 100 μM histamine. The inositol phosphate production was only blocked by an H_1 antagonist and not by an H_2 nor H_3 antagonist. In addition, this cell line, expressing a high density of H_1 receptors (861 ± 41 fmol/mg of protein) allows for the first time investigation of regulatory phenomena such as receptor downregulation. As such, the CHOhumH_1 cell line may be considered a suitable model system to study H_1 receptor regulation and signalling.

The recent cloning of the guinea-pig (Horio *et al.*, 1993, Traiffort *et al.*, 1994), bovine (Yamashita *et al.*, 1991), rat (Fujimoto *et al.*, 1993) and human H_1 receptor (De Backer *et al.*, 1993, Fukui *et al.*, 1994, Moguilevsky *et al.*, 1994) has revealed several potential phosphorylation sites in the amino acid sequence of the H_1 receptor. Three potential PKC phosphorylation sites are found in the amino acid sequence of the H_1 receptor gene, as well as a potential protein kinase A site. In addition, the third intracellular loop contains many threonine and serine residues that can be potentially phosphorylated by specific receptor kinases. As it is well-known for the family of G-protein coupled receptors, that kinases play important roles in mechanisms such as receptor desensitization and downregulation (Collins, 1993, Lohse, 1993), all these sites may represent possible targets for phosphorylation.

Previous studies have shown that the histamine H_1 receptor is susceptible to short-term receptor desensitization. Depending on the cell type both homologous (Cowlen *et al.*, 1990, Dickenson and Hill, 1993, Dillon-Carter and Chuang, 1989, Leurs *et al.*, 1990, McCreath *et al.*, 1994, Quach *et al.*, 1981, Smit *et al.*, 1992) and heterologous (Bristow and Zamani, 1993, Brown *et al.*, 1986, Dickenson and Hill, 1993, McDonough *et al.*, 1988) H_1 receptor desensitization have been observed. In the present study it was shown that short-term exposure of CHOhumH_1 cells to histamine resulted in a decrease of subsequent histamine-induced Ca^{2+} responses. The histamine-induced desensitization appeared to be heterologous as the ATP-induced Ca^{2+} response, mediated by interaction with endogenous purinergic P_{2U} receptors present on this cell line (Iredale and Hill, 1993, Leurs *et al.*, 1994), was also found to be affected. Moreover, pretreatment of CHOhumH_1 cells with ATP also desensitized the histamine-induced Ca^{2+} responses.

The process of heterologous histamine-induced desensitization in CHOhumH_1 cells can be ascribed to an alteration at the level of the intracellular Ca^{2+} pool, as reported previously for other model systems (Hishinuma and Uchida, 1988, Leurs *et al.*, 1990, McDonough *et al.*, 1988). The Ca^{2+} response of caffeine, which releases Ca^{2+} from intracellular Ca^{2+} stores (Palade *et al.*, 1989) was attenuated upon short-term histamine exposure. Ineffective Ca^{2+}-pump or Ca^{2+} release mechanisms of the intracellular Ca^{2+} pool might explain the observed findings.

In various cellular systems PKC has been found to be implicated in negative feedback of H_1 receptor signalling (Cowlen *et al.*, 1990, Dickenson and Hill, 1993, Dillon-Carter and

Chuang, 1989, Fukui *et al.*, 1991, Leurs *et al.*, 1990, McCreath *et al.*, 1994, Smit *et al.*, 1992). Besides attenuation of H_1 receptor signalling, direct activation of PKC was also found to desensitize other phospholipase C-linked receptors present on these cellular systems, implying that PKC induces heterologous desensitization. In DDT_1MF-2 cells, however, PKC-independent mechanims appeared to be involved in the heterologous desensitization of the histamine H_1 and ATP receptor-mediated Ca^{2+} responses (Dickenson and Hill, 1993). In $CHOhumH_1$ cells the PKC activator, PMA, was found to inhibit the histamine- as well as the ATP-induced Ca^{2+} reponse. At a maximally effective concentration of PMA (Leurs *et al.*, 1994), the inhibition of the response was only partially and clearly less effective than histamine-pretreatment. In contrast, in e.g. cultured astrocytes (Fukui *et al.*, 1991), human umbilical vein endothelial cells (McCreath *et al.*, 1994) and in HeLa cells (Smit *et al.*, 1992) H_1 receptor mediated responses have been reported to be highly sensitive to PKC activation. Moreover, in $CHOhumH_1$ cells PKC downregulation induced by long-term exposure to PMA did not affect the histamine-induced desensitization nor did pretreatment with the PKC inhibitor staurosporine. These results indicate that in $CHOhumH_1$ cells PKC is probably not involved in the heterologous desensitization.

The discrepancy regarding the role of PKC in the heterologous desensitization between $CHOhumH_1$ cells and other cellular systems may be explained by differential expression of PKC isoenzymes. PKC is known to consist of a family of different PKC isoenzymes (Nishizuka, 1988). As such, this cell line may be considered as a suitable model system to investigate the role of different PKC isoenzymes in the negative feedback modulation of the human H_1 receptor more closely. Therefore, we stably expressed different PKC isoenzymes cDNAs (α, β_I, β_{II}, δ, ε and γ) (Ono *et al.*, 1987) separately into $CHOhumH_1$ cells. Although clonal cell lines, expressing 1.4 to 3.4 fold [^{3}H]-PdBu binding were obtained (data not shown), [^{3}H]-mepyramine binding studies revealed disappearance of H_1 receptors on membranes of these clonal $CHOhumH_1$ cell lines, overexpressing one of the PKC isoenzymes. Since our co-expression approach was unsuccessful, a detailed knowledge of the distribution of different PKC isoenzymes in the various cellular systems, as well as the future availibity of specific PKC isoenzyme inhibitors/activators, could perhaps give more insight in the contribution of each PKC isoenzyme in the negative feedback of H_1 receptor signalling.

Thus far, no mechanistic information is available on long-term regulatory mechanisms such as H_1 receptor downregulation. Long-term treatment of $CHOhumH_1$ cells with histamine or other H_1 agonists resulted in a time- and dose-dependent decrease in the number of H_1 receptor binding sites. In the neuroblastoma cell line NIE-115 and neuroblastoma/glioma hybrid cell line NG108-15, PKC stimulation with phorbolesters was found to induce downregulation of respectively muscarinic receptors (Liles *et al.*, 1986) and δ-opioid receptors (Gucker and Bidlack, 1992). Yet, neither short-term nor long-term stimulation of the $CHOhumH_1$ cells with PMA did affect H_1 receptor expression. For the δ-opioid receptor

Gucker and Bidlack showed that simultaneous activation of PKC and the δ-receptor is required to induce receptor downregulation (Gucker and Bidlack, 1992). Yet, no downregulation was observed when cells were treated simultaneously with PMA and histamine for a period of 4 hrs, indicating that PKC potentiation is apparently not occuring in CHOhumH$_1$ cells. In addition, concurrent incubation of cells with PMA and histamine for 24 hrs did not induce a more pronounced H$_1$ receptor downregulation than with histamine alone.

No significant H$_1$ receptor downregulation in CHOhumH$_1$ cells was observed upon long-term ATP exposure, indicating that the H$_1$ receptor density is not affected upon activation of other receptors coupled to phospholipase C. Thus, stimulation of phospholipase C is not sufficient to induce a reduction of receptor binding sites. These findings are corroborated by the difference in potency for histamine-induced inositol phosphate production and receptor downregulation (EC_{50} 2.23 μM and 0.13 μM, respectively). Interestingly, the EC_{50} for the homologous short-term desensitization of the H$_1$ receptor in human umbilical vein endothelial cells was also found to be lower than the EC_{50} for inositol phosphate production (McCreath *et al.*, 1994). Taken together, these data imply that H$_1$ receptor downregulation is apparently not induced by activation of phospholipase C or PKC.

To study the possible functional consequences of the H$_1$ receptor downregulation, we also measured the Ca^{2+} responses in histamine-downregulated CHOhumH$_1$ cells. We observed that long-term histamine exposure of CHOHumH$_1$ cells also affected receptor mediated Ca^{2+} signalling. Long-term treatment of CHOhumH$_1$ cells to 100 μM histamine resulted in attenuation of both the histamine- as well as the ATP-induced Ca^{2+} responses, suggesting that the reduction of the Ca^{2+} responses is not only caused by H$_1$ receptor downregulation. If PKC stimulation would be responsible for the effects of long-term stimulation on the Ca^{2+} responses, one would expect based on the partial sensitivity of the Ca^{2+} responses towards PMA that Ca^{2+} responses of histamine-pretreated (24 hrs) cells would not be further affected by PMA treatment. Nevertheless, PMA still induced a similar reduction of the histamine-induced Ca^{2+} response in CHOhumH$_1$ cells pretreated for 24 hrs with histamine, indicating that PKC is not responsible for the observed reduction of the histamine and ATP mediated Ca^{2+} responses. Although we have no direct evidence, this reduction may be explained by an alteration at a level distant from the receptor, such as the G-protein or Ca^{2+} pool. Mullaney *et al.* (1993) showed that agonist-induced downregulation of m$_1$ muscarine acetylcholine receptors expressed in CHO cells was paralleled by a specific downregulation of cellular levels of the α subunits of G-protein G_q and G_{11}. For other receptor systems, linked to adenylate cyclase, similar observations were made with regard to $G_{\alpha s}$ subunit downregulation (see for references Mullaney *et al.*, 1993). Another possibility is a downregulation of the IP_3 receptor upon long-term receptor activation. In SH-SY5Y human neuroblastoma cells, chronic muscarinic stimulation was found to suppress the Ca^{2+} releasing activity of IP_3, which was paralleled by a reduction in the number of IP_3 binding sites (Wojcikiewicz *et al.*, 1992).

In conclusion, human H$_1$ receptors expressed into CHO cells are susceptible to receptor

regulation. Short-term activation of the human H_1 receptor to histamine leads to an heterologous desensitization of the agonist-induced Ca^{2+} response, which may be explained by alterations at the level of the intracellular Ca^{2+} pool. Long-term exposure of the CHOhumH_1 cells results in a dose- and time-dependent downregulation of the human H_1 receptor. Both histamine and ATP-induced Ca^{2+} responses are affected upon long-term histamine treatment, indicating alterations at a level distant from the receptor. PKC does not seem to play a role in either the histamine-induced H_1 receptor desensitization or downregulation in CHOhumH_1 cells, which may be explained by differential expression of PKC isoenzymes in this cell line compared to other cellular systems.

Further detailed investigations are required to elucidate the biological mechanism underlying H_1 receptor downregulation. Other phospholipase C-linked receptors, such as the muscarinic m1 receptor, are also downregulated upon long-term agonist exposure (Shapiro and Nathanson, 1989). Studies have shown that the third intracellular loop is important for the agonist-induced muscarine m1 receptor downregulation. The future use of mutant H_1 receptors, should give more insight in the structural requirements of H_1 receptor desensitization and downregulation.

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Chapter 4

Rapid desensitization of the histamine H_2 receptor on the human monocytic cell line U937

M. J. Smit, R. Leurs, S. R. Shukrula, A. Bast and H. Timmerman

In the present study we have subjected the histamine H_2 receptor on the monocytic cell line U937 to a thorough pharmacological characterization using a series of selective histamine H_1, H_2 and H_3 receptor agonists and antagonists. Recent reports have demonstrated the existence of a histamine H_2 receptor on HL-60 and HGT-1 cells with a pharmacological profile distinct from the commonly described histamine H_2 receptor (Burde *et al.*, 1989, Mitsuhashi *et al.*, 1991, Seifert *et al.*, 1992b). U937 cells, however, seem to express classical histamine H_2 receptors. Histamine and dimaprit dose dependently induce the formation of cAMP, whereas dimaprits inactive analogues, nordimaprit and homodimaprit, show reduced potenties. Histamine H_1 and H_3 receptor agonists do not show histamine H_2 receptor activity. Various histamine H_2 receptor antagonists are able to block the histamine-induced production of cAMP with an antagonistic profile comparable to that observed in the guinea-pig right atrium.

Furthermore, endogenous histamine H_2 receptors in U937 cells are found to be susceptible to receptor desensitization, a mechanism which may become apparent under pathophysiological conditions or during drug therapy. A 30 mins pre-exposure of U937 cells to histamine (100 μM) results in a 50% attenuation of the production of cAMP by a subsequent application of agonist. Desensitization of the histamine H_2 receptor in U937 cells is found to be homologous as the β-adrenoceptor mediated response remains unaffected.

Introduction

Histamine is a widely occurring endogenous compound, known among others as an inflammatory mediator, a factor involved in cardiac and gastrointestinal regulation and a neuroregulator (Leurs *et al.*, 1991, Schwartz and Haas, 1992). Pharmacological analyses, supported by the availability of selective ligands, have demonstrated the existence of three major subclasses of histamine receptors, designated as H_1, H_2 and H_3 (Leurs *et al.*, 1991, Schwartz and Haas, 1992). The histamine H_2 receptor, present in the heart, stomach and brain, has been shown to be associated with a G-protein mediated stimulation of adenylate cyclase, causing an increase in cAMP (Hill, 1991). Histamine H_2 receptor antagonists have been proven to be successful in the therapy of gastric ulcers (Bertaccini and Corruzi, 1992). Recently, also the application of cardioselective histamine H_2 receptor agonists during congestive heart failure has been suggested (Felix *et al.*, 1991).

Under several pathophysiological conditions, e.g. inflammation, histamine is released from either mast cells or basophils in high quantities. It is conceivable that histamine receptors present at the site of inflammation may be affected and become desensitized. Desensitization is a general occurring phenomenon observed for almost all G-protein coupled receptors. It is characterized by an attenuation of the cellular response despite the presence of the agonist. This phenomenon might have serious consequences during various physiological and pathophysiological processes. Moreover, it may also become apparent during drug therapy

with agonists (Brodde *et al.*, 1990, Motomura *et al.*, 1990, Ghosh *et al.*, 1990).

As for the histamine H_1 receptors, we and others have demonstrated that these receptors are rapidly desensitized (see for references Smit *et al.*, 1992). In contrast, few reports have described desensitization of the histamine H_2 receptor (Chan *et al.*, 1982, Johnson and Sawutz, 1984, Prost *et al.*, 1984, Schreurs *et al.*, 1984). In view of the anticipated use of histamine H_2 receptor agonists during cardiac heart failure (Felix *et al.*, 1991) detailed knowledge of histamine H_2 receptor regulation seems to be essential for the development of effective drug therapy. One of the underlying mechanisms for the development of cardiac heart failure was suggested to be β-adrenoceptor desensitization (Stiles, 1991).

Potential use of histamine H_2 receptor agonists during inflammatory processes has also been suggested (Plaut and Lichtenstein, 1982). The second messenger cAMP is thought to exert an inhibitory effect on the activity of immune and inflammatory cells through prevention of antigen-induced release of histamine from leukocytes (Bourne *et al.*, 1974, Kammer, 1988, Torphy *et al.*, 1992). Thus, under pathophysiological conditions activation of histamine H_2 receptors may prevent further release of histamine, leading to an attenuation of the inflammatory process. However, contributions of the histamine H_2 receptors to this effect could be limited by the occurrence of receptor desensitization.

In order to examine whether endogenous histamine H_2 receptors are susceptible to desensitization we looked for a suitable model system. Due to limited availability of human tissue we preferred to use an isolated cellular system. The promyelocytic cell line HL-60 (Gespach *et al.*, 1982), the monocytic leukemic cell line U937 (Gespach *et al.*, 1985), and the gastric carcinoma cell lines HGT-1 (Emani *et al.*, 1983) and MKN-45 (Arima *et al.*, 1991), all of human origin, can be used as a model system for the human histamine H_2 receptor. Yet, recent reports have implied the existence of a histamine H_2 receptor on the HL-60 (Burde *et al.*, 1989, Mitsuhashi *et al.*, 1991, Seifert *et al.*, 1992b) and HGT-1 cell line (Reyl-Desmars *et al.*, 1991) with a pharmacological profile distinct from the commonly described histamine H_2 receptor. These findings prompted us to use the U937 cell line. As the histamine H_2 receptor had been moderately characterized on the U937 cell line (Gespach *et al.*, 1985), we first subjected the histamine H_2 receptor to a thorough pharmacological characterization. Histamine H_2 receptor activity and specificity were evaluated by means of measurements of cAMP levels in these cells, using a series of selective histamine H_1, H_2 and H_3 receptor agonists and antagonists. Thereafter we investigated whether the histamine H_2 receptor is susceptible to receptor desensitization.

Materials and methods

Cell culture U937 cells, obtained from the American Type Culture Collection, were cultured at 37°C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal-calf serum (GIBCO), 2 mM L-glutamine, 50 IU /ml penicillin and 50 µg/ml streptomycin (GIBCO). Culture medium was changed every 3 days.

cAMP assay Pretreatment of cells (desensitization experiments) with histamine was performed in RPMI at

37°C in a humidified atmosphere with 5% CO_2. Cells were washed and resuspended in Krebsbuffer supplemented with 1% bovine serum albumine and 1 mM isobutylmethylxanthine (IBMX) at a cell density of 10^6 cells/ml. Thereafter cells were preincubated for 10 mins at 37°C and exposed to various drugs for 10 mins at the indicated concentrations. The reaction was stopped by addition of $HClO_4$ (6 N) and cells were stored at ice for 30 mins. Thereafter cells were centrifuged at 3000 x g for 10 mins. The supernatant was neutralized using freon/ trioctylamine (1:1) and centrifuged at 3000 x g for 10 mins. The cAMP content (supernatant) was determined by means of competition with [^{3}H]-cAMP for protein kinase A, according to the method described by Nordstedt and Fredholm (1990). Briefly, a protein kinase A containing fraction was isolated from bovine adrenal glands. Adrenal cortex was homogenized in 10 volumes of 100 mM Tris/HCl, 250 mM NaCl, 10 mM EDTA, 0.25 M sucrose and 0.1% 2-mercaptoethanol (pH = 7.4 at 4°C, buffer A) using a Omni-Sorval mixer (30 s, maximal speed) and a Polytron homogenizer (10 s, maximal speed). The homogenate was centrifuged for 60 mins at 30000 g at 4°C. The supernatant, containing protein kinase A, was carefully recovered and frozen in 1 ml aliquots at -80°C. Before use, the binding protein was diluted five-fold in ice-cold buffer A without sucrose and 2-mercaptoethanol and kept on ice. Subsequently, 200 µl of the binding protein was mixed with 100 µl of the U937 homogenate or cAMP standards and 30.000 dpm [^{3}H]-cAMP. After incubation for 150 mins at 4°C the mixture was rapidly diluted with 3 ml ice-cold 50 mM Tris-HCl (pH 7.4, at 4°C) and filtered through Whatman GF/B filters using a Brandel cell-harvester (Semat, UK). The radioactivity retained on the filters was measured by liquid scintillation counting.

Chemicals Histamine dihydrochloride, forskolin, isoprenaline, isobutylmethylxanthine (IBMX), cAMP and mepyramine were obtained from Sigma Chemical Company Ltd. (St. Louis, MO, U.S.A.). 1,1,2-trichlorotrifluoroethane (freon) was purchased from Aldrich Chemical Company. Tri-n-octylamine was purchased from Janssen Chimica. [^{3}H]-cAMP (40 Ci/mmol) was obtained from Amersham. Dimaprit dihydrobromide, nor- and homodimaprit dihydrobromide, impromidine trihydrobromide, amthamine dihydrobromide, amselamine dihydrobromide, 2-pyridylethylamine and thioperamide were taken from laboratory stock.

Gifts of burimamide, metiamide, cimetidine (SmithKline Beecham), tiotidine (Imperial Chemical Industries), ranitidine (Glaxo), famotidine (Merck Sharp & Dohme), mifentidine (De Angeli) and R(α)-methylhistamine dihydrobromide (Dr. J.C. Schwartz, Paris) are acknowledged. All other chemicals were of analytical grade.

Data analysis The cAMP production is depicted in absolute values as pmol cAMP produced by 10^6 cells or represented as % stimulation compared to the maximum-induced response of 100 µM histamine. Antagonistic properties of the histamine H_2 receptor antagonists towards the histamine H_2 receptor were analyzed assuming competitive inhibition, according to the Cheng-Prusoff (1973) equation: $K_i = IC_{50}/(1 + L/EC_{50})$ where K_i is the inhibition constant of the antagonist, the EC_{50} is the dose required to induce 50% of the maximal attained response, the IC_{50} the dose required for 50 % inhibition produced by histamine (100 µM) and L the concentration of the ligand, histamine. All data shown are expressed as mean ± s.e.mean of at least three independent experiments. Statistical analysis was carried out using Student's t-test. P values < 0.05 indicate a statistically significant difference.

Results

Histamine-induced cAMP production in U937 cells

Addition of 100 µM histamine induced a rapid and significant increase of the production of cAMP in time in the presence of 1 mM of the phosphodiesterase inhibitor IBMX in U937 cells (Fig. 1A). Already 1 min after application of 100 µM histamine significant changes in the cellular cAMP levels were measured (Fig. 1A). A maximal response (81 ± 7 pmol/10^6 cells, an approximately 11 fold increase of cAMP compared to basal cAMP levels) was observed after 10 mins of incubation of the cells with histamine. No additional production of cAMP was observed when cells were exposed to histamine for longer incubation periods. The cAMP production in U937 cells was dose dependently increased when the cells were incubated for 10 mins with increasing concentrations of histamine (Fig. 1B). Half maximal stimulation (EC_{50}) was observed at 13 ± 0.1 µM of histamine (Table 1).

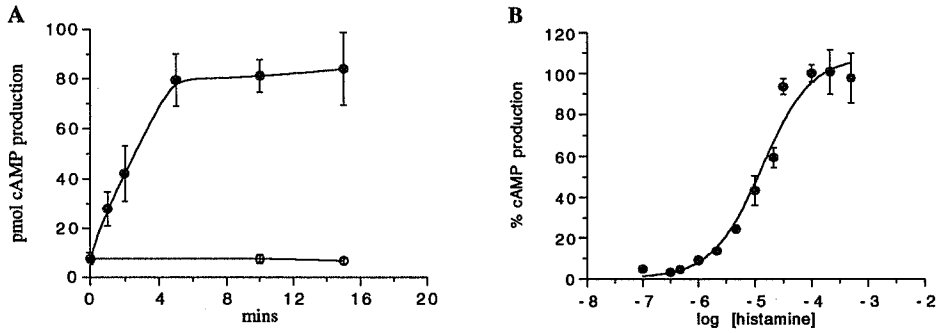

Fig. 1. Histamine induced cAMP accumulation in U937 cells. A, U937 cells were stimulated with (filled circles) or without (open circles) histamine (100 μM) at 37 °C in Krebs buffer supplemented with 1 mM IBMX for various length of time. B, U937 cells were incubated with increasing concentrations of histamine for 10 mins at 37°C in Krebs buffer supplemented with 1 mM IBMX and 1% bovine serum albumin (basal level 7.4 ± 1.2 pmol/10^6 cells, maximal induced response by histamine (100 μM) 85 ± 6 pmol/10^6 cells). Data shown are the mean values of triplicate experiments.

Pharmacological characterization of the histamine H_2 receptor on U937 cells

A wide range of histamine H_1, H_2 and H_3 receptor agonists and antagonists was used to subject the histamine H_2 receptor on U937 cells to a pharmacological characterization (Fig. 2, 3 and Table 1). Dimaprit, a selective histamine H_2 receptor agonist was able to induce a dose dependent increase of cAMP (Fig. 2). The maximum production of cAMP induced by dimaprit was comparable to the increase of cAMP induced by histamine (Table 1). The pD_2 of dimaprit was 4.79 ± 0.12 μM. Yet, dimaprit's structural analogues homodimaprit and nordimaprit, which are devoid of histamine H_2 receptor agonist activity (Sterk *et al.*, 1986), failed to induce a response comparable to that induced by dimaprit (Fig. 2). The pD_2 's of the latter compounds were also markedly reduced. The respective histamine H_1 and H_3 receptor agonists, 2-pyridylethylamine and R(α)-methylhistamine, showed weak histamine H_2 receptor agonistic effects only at high concentrations (Table 1).

A wide range of histamine H_2 receptor antagonists dose dependently inhibited the histamine-induced cAMP response (Fig. 3A). Their K_i values are depicted in table 1. In order to determine whether the antagonists show similar potencies for the histamine H_2 receptor located on U937 cells as well as guinea-pig heart atrium, the K_i values on both systems were compared. As can be seen in table 1 the pK_i values of the different antagonists showed a good correlation (r = 0.89) with the reported pA_2 values against the histamine-induced chronotropic response of the guinea-pig right atrium (Fig. 3B) (Leurs *et al.*, 1991).The histamine H_1 and H_3 receptor antagonist, respectively mepyramine and thioperamide, did not show any antagonistic activity, up to 10 μM, towards the response elicited by histamine (Table 1).

Table 1 pK_i values of various drugs for inhibition of the histamine (100 μM) induced cAMP response. Reported pA_2 values for guinea-pig right atrium are shown for comparison. pD_2 and E_{max} values of histaminergic agonists. Data of the E_{max} values are shown as a percentage of the histamine (100 μM) induced response.

Drugs	U937 cells pK_i	guinea-pig right atrium pA_2
Antagonists		
burimamide	5.44 ± 0.14	5.11
cimetidine	5.64 ± 0.07	6.10
metiamide	6.15 ± 0.18	6.04
ranitidine	6.89 ± 0.13	7.20
tiotidine	7.40 ± 0.30	7.82
famotidine	7.74 ± 0.13	7.60
mifentidine	8.17 ± 0.15	7.76
mepyramine	< 5	
thioperamide	< 5	
	pD_2	E_{max} (%)
Agonists		
histamine	4.89 ± 0.10	100 ± 9
dimaprit	4.79 ± 0.12	117 ± 20
nordimaprit	< 4	43 ± 5 *
homodimaprit	< 4	9 ± 2 *
2-pyridylethylamine	< 4	-
R(α)-methylhistamine	< 4	-

* E_{max} value determined at 1 mM.

Histamine H_2 receptor desensitization

In order to investigate whether endogenous histamine H_2 receptors on this cell line are susceptible to receptor desensitization the cells were exposed to 100 μM of histamine for periods ranging from 0.5 to 2 hours in the absence of IBMX. Thereafter the cells were washed and exposed to 100 μM of histamine in the presence of 1 mM of IBMX for 10 mins to determine whether the histamine H_2 receptors were still able to generate cAMP. As can be seen in figure 4A, impaired production of cAMP induced by histamine is observed when pre-exposure time of U937 cells to histamine increases. A reduction of 35 % of the histamine-induced cAMP response is found when cells are pretreated with 100 μM of histamine for 30 mins. After 1 hr pretreatment of cells with histamine, only a minor response is observed (9 ± 4 %). Histamine can no longer induce a response when cells are pretreated with 100 μM histamine for 120 mins. Half maximal desensitization is observed after approximately 35 mins pre-exposure of cells to histamine. Exposure of cells to increasing concentrations of histamine for 1 hr led to a dose dependent decrease of the histamine H_2 receptor mediated cAMP response with an EC_{50} value of 0.67 ± 0.21 μM (Fig. 4B).

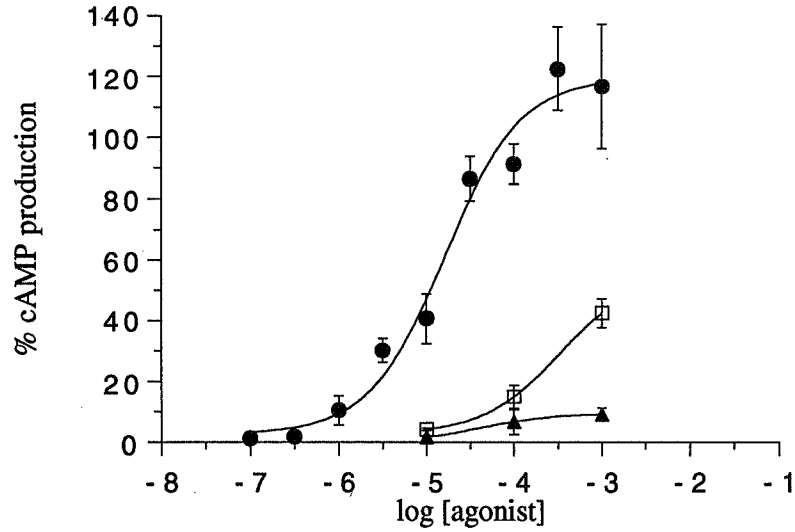

Fig. 2. Dose dependent increase of the cAMP production by dimaprit (filled circles), nordimaprit (open squares) and homodimaprit (filled triangles). Data are shown as a percentage of the histamine (100 μM) induced response (85 ± 6 pmol/10^6 cells) over basal levels (7.4 ± 1.2 pmol/10^6 cells). U937 cells were incubated with the indicated drugs with increasing concentrations for 10 mins at 37°C in Krebs buffer in the presence of 1 mM IBMX and 1% bovine serum albumin. Data shown are the mean values of triplicate experiments.

To define the specificity of the histamine-induced desensitization we performed some experiments with forskolin, which directly activates adenylate cyclase. In U937 cells the cAMP production was dose dependently increased by forskolin (data not shown). Addition of 10 μM of forskolin resulted in cAMP production which was 5 times higher than the histamine-induced cAMP production. As can be seen in fig. 4B the forskolin-induced production of cAMP remains unaffected after respectively 30 and 60 mins pretreatment of cells with 100 μM of histamine.

In order to establish whether the observations of receptor desensitization were confined to the histamine H_2 receptor, we examined the effects of histamine pretreatment on the β-adrenoceptor which is also present on U937 cells (Chan *et al.*, 1982). Isoprenaline, a β-adrenoceptor agonist, dose dependently increased the cAMP production (data not shown). Exposure of cells to 10 μM of isoprenaline lead to an increase in cAMP, which was 3 times higher than the histamine-induced production of cAMP. Fig. 4B shows a unaltered generation of cAMP after histamine-induced desensitization, indicating that histamine H_2 receptor desensitization is restricted to the histamine H_2 receptor.

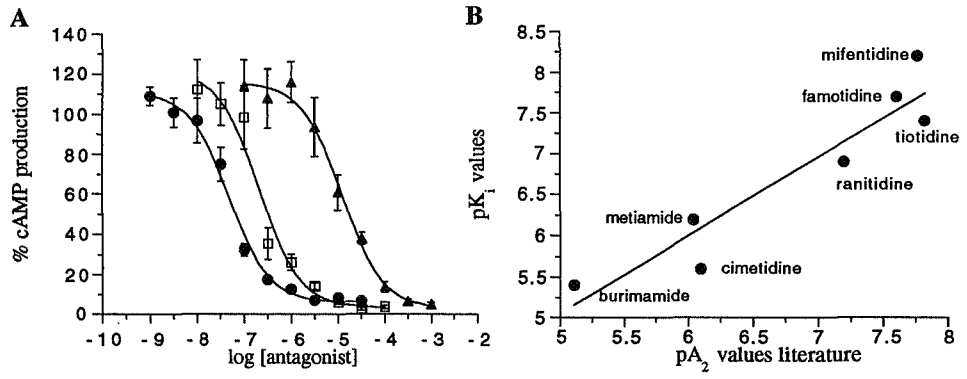

Fig. 3. A, Pharmacological characterization of the histamine induced cAMP response in U937 cells. Cells were incubated for 10 mins at 37°C with 100 μM of histamine in the presence of increasing concentrations of mifentidine (filled circles), tiotidine (open squares) and cimetidine (filled triangles). Data are shown as a percentage of the histamine (100 μM) induced response (respectively 88 ± 5, 91 ± 15, 92 ± 14 pmol/10^6 cells) over basal levels (7.0 ± 1.6 pmol/10^6 cells). Data shown are the mean values of triplicate experiments. B, Comparison of pK$_i$'s values of various drugs for inhibition of the histamine (100 μM) induced cAMP response on U937 cells and reported pA$_2$ values of these drugs on guinea-pig heart atrium (Leurs et al., 1991).

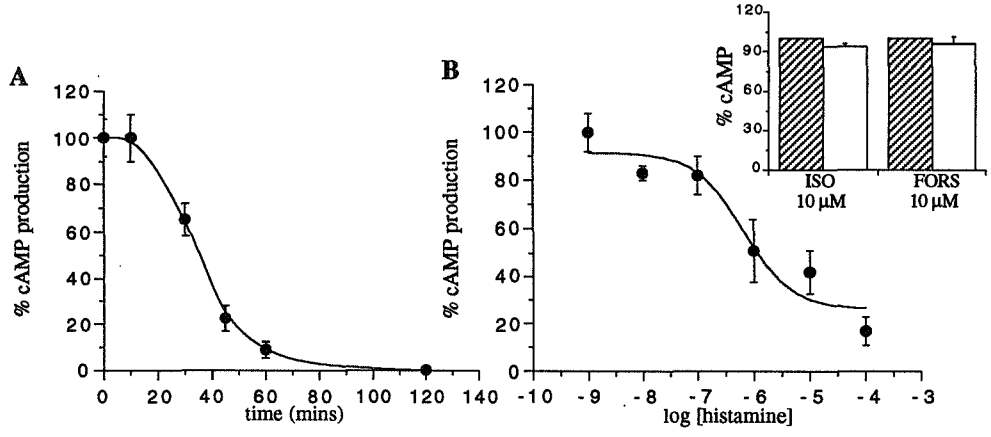

Fig. 4. Histamine induced desensitization of the histamine H_2 receptor. A, U937 cells were incubated for various length of time with 100 μM of histamine. Thereafter cells were washed and stimulated with 100 μM of histamine for 10 mins. Data are shown as a percentage of the histamine (100 μM) induced response (79 ± 7 pmol/10^6 cells) over basal levels (5.9 ± 2.1 pmol/10^6 cells) in non pretreated cells. B, U937 cells were incubated for 1 hr with increasing concentrations of histamine, washed and stimulated with 100 μM of histamine for 10 mins. Data are shown as a percentage of the histamine (100 μM) induced response (98 ± 7 pmol/10^6 cells) over basal levels (6.0 ± 2.0 pmol/10^6 cells) in non pretreated cells. Data shown are the mean values of triplicate experiments. (Inset) Effect of histamine H_2 receptor desensitization on the β-adrenoceptor and forskolin induced accumulation of cAMP. U937 cells are preincubated for 60 mins with (open columns) and without (hatched columns) 100 μM of histamine, washed and stimulated with 10 μM of isoprenaline (ISO) and 10 μM forskolin (FORS). Data are shown as a percentage of respectively the isoprenaline (10 μM, 244 pmol/10^6 cells) and forskolin (10 μM, 407 pmol/10^6 cells) induced response in non pretreated cells.

Discussion

The use of isolated cellular systems derived from human tissue for a detailed investigation of regulatory mechanisms is now quite current. Human cell lines represent a simplified model system consisting of a homogenous cell population. For the histamine H_2 receptor one often uses the human leukemic cells HL-60, a promyelocytic cell line (Gespach *et al.*, 1982), the U937 (Gespach *et al.*, 1985), a monocytic cell line, and gastric cell lines HGT-1 (Emani *et al.*, 1983) and MKN-45 (Arima *et al.*, 1991). All cell lines show production of cAMP upon addition of histamine. Yet, the pharmacological profiles of these reported histamine H_2 receptors were shown be distinct from the commonly described histamine H_2 receptor (Burde *et al.*, 1989, Mitsuhashi *et al.*, 1989, Reyl-Desmars *et al.*, 1991, Seifert *et al.*, 1992b).

In HL-60 cells histamine was also found to induce rises of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) besides the increase in production of cAMP (Mitsuhashi *et al.*, 1989, Seifert *et al.*, 1992b). Surprisingly, the histamine-induced rise in $[Ca^{2+}]_i$ appeared to be primarily mediated via histamine H_2 receptors rather than histamine H_1 receptors. Moreover, the agonist/antagonist profile of the histamine H_2 receptor mediated rise in $[Ca^{2+}]_i$ did not correspond to the one observed for the induced cAMP production (Seifert *et al.*, 1992b), suggesting the existence of different histamine H_2 receptor subtypes. Moreover, Burde *et al.*, (1989) also described a unique pharmacological profile for the inhibition of the oxidative burst in these cells. Recently, Seifert *et al.* (1992a) reported the possible existence of histamine H_1 receptors on these cells. There are numerous reports that have shown that histamine H_1 receptor agonists appear to potentiate the cAMP response of receptors coupled to adenylate cyclase (see for references, Johnson *et al.*, 1992). It is therefore not clear whether the H_2 induced response is affected by the presence of histamine H_1 receptors. Also in the HGT-1 tumour cell line the pharmacological profile of the histamine H_2 receptor appeared to be quite distinct from the commonly described histamine H_2 receptor system; tiotidine showed a rather low affinity for the histamine H_2 receptor (Reyl-Desmars *et al.*, 1991). Based on these observations we concluded that both the HL-60 and HGT-1 cells are not suitable for detailed investigation of histamine H_2 receptor regulation. Moreover, little information is available on the pharmacological profile of the histamine H_2 receptor on U937 (Gespach *et al.*, 1985) and MKN-45 cells (Arima *et al.*, 1991). Therefore we examined the histamine H_2 receptor on U937 cells in more detail.

In view of our findings, U937 cells seem to express classical histamine H_2 receptors. Dimaprit, a selective histamine H_2 receptor agonist, is as potent as histamine in inducing formation of cAMP. Yet, its structural analogues nordimaprit and homodimaprit were less effective in generating cAMP and showed reduced potencies. Variation in chain length by one methyl group (nordimaprit and homodimaprit) leads to a loss of histamine H_2 receptor activity (Sterk *et al.*, 1986). Moreover, initial experiments with the histamine H_2 receptor agonists amthamine and amselamine, which appear to be potent histamine H_2 receptor agonists (Eriks *et al.*, 1992, Van der Goot *et al.*, 1994), have shown that these new selective histamine H_2

receptor agonists are effective in this model system as well (M.J. Smit, unpublished observation). In addition, the histamine H_1 and H_3 receptor agonists, 2-pyridylethylamine and R(α)-methylhistamine, showed weak histamine H_2 receptor agonistic effects only at high concentrations.

Various histamine H_2 receptor antagonists were able to block the histamine-induced production of cAMP. On the other hand the histamine H_1 and H_3 receptor antagonists, mepyramine and thioperamide, respectively, failed to inhibit the effect induced by 100 μM histamine at concentrations up to 10 μM. The different histamine H_2 receptor antagonists showed an antagonistic profile in U937 cells comparable to that observed in the guinea-pig right atrium, the commonly accepted histamine H_2 receptor reference system. Moreover, Seifert et al. (1992b) have shown that addition of histamine in these cells does not lead to an increase in $[Ca^{2+}]_i$ in U937 cells; cAMP responses are therefore not influenced by cross-talk with the Ca^{2+} signalling pathways. Taken together, the U937 cells provide a good model system for the investigation of human histamine H_2 receptor regulation.

Limited information is available on desensitization of the histamine H_2 receptor. In the present study we have found that histamine H_2 receptors were susceptible to desensitization. The histamine H_2 receptor mediated cAMP response was found to be time and dose dependently impaired when cells were pretreated with histamine (100 μM). The half time for desensitization was 35 mins. An almost complete reduction of the histamine-induced response was observed after 1 hr incubation of the cells with histamine. The mechanism remains restricted to the level of G-protein or receptor protein as forskolin was still able to induce a response after exposure of cells to histamine. U937 cells also contain β-adrenoceptors (Torphy *et al.*, 1992), which couple to the adenylate cyclase pathway. Desensitization of the histamine H_2 receptor seemed to be homologous as the β-adrenoreceptor mediated responses remained unaffected after pretreatment with histamine.

Desensitization of the histamine H_2 receptor has been reported previously, but the mechanism responsible for the loss of receptor responsiveness remains to be elucidated. In HL-60 cells desensitization of putative histamine H_2 receptors has been shown to be mainly homologous, which can not be explained by a loss of receptors nor by the induction of phosphodiesterase activity (Johnson and Sawutz, 1984). In some experiments prostaglandin E_2 responses were affected, indicating a small heterologous component (Johnson and Sawutz, 1984). The half time of desensitization of the histamine H_2 receptor in the HL-60 cell line appeared to be about 2.5 h (Johnson and Sawutz, 1984). In clonal cytolytic T lymphocytes and human gastric cancer cell line, HGT-1, histamine (50 μM and 1 mM) induced desensitization with a half-time of 10 mins and 20 mins respectively (Schreurs *et al.*, 1984, Prost *et al.*, 1984). Yet, since the actual pharmacological profile of the histamine receptor is undefined in the HL-60 and HGT-1 cell lines, these results do not necessarily reflect regulation of the the classical histamine H_2 receptor.

In human mononuclear leukocytes histamine caused a heterologous desensitization of the H_2, β- and prostaglandin E_2 receptor, which could be explained by enhanced phosphodiesterase activity (Chan *et al.*, 1982, Holden *et al.*, 1987). Also in U937 cells the

β-adrenoceptor-induced cAMP production was found to be reduced after prolongued exposure (hours) of cells to a β-agonist. This reduction could also be explained by an increase of phosphodiesterase activity (Torphy *et al.*, 1992). Since this increased phosphodiesterase activity was only observed after 3 hours pretreatment, it is therefore unlikely that this effect may account for the observed histamine-induced histamine H_2 receptor desensitization in U937, which has a half time of 30 mins. Moreover, forskolin and the β-adrenoceptor mediated cAMP production were not affected, excluding an increased phosphodiesterase activity as explanation for the observed histamine H_2 receptor desensitization.

The mechanism of desensitization of adenylate cyclase-linked receptors has been characterized best for the β-adrenoceptor (Hausdorff *et al.*, 1990, Huganir and Greengard, 1990). Desensitization is associated with receptor phosphorylation catalyzed by a specific kinase called β-adrenoceptor kinase, which specifically phosphorylates the agonist-occupied form of the receptor. This kinase is responsible for homologous desensitization. The other kinase is the cAMP dependent kinase (protein kinase A), which is responsible for heterologous desensitization. Besides β-adrenoceptor kinase, another cofactor, called βarrestin, has been established to be involved in the process of desensitization (Lohse *et al.*, 1990). In the meantime other G-protein coupled receptors were also found to be substrates for β-adrenoceptor kinase (Benovic *et al.*, 1989, Lefkowitz *et al.*, 1990). To date, up to 6 distinct cDNAs encoding β-adrenoceptor kinase isoenzymes, currently referred to as G-protein coupled receptor kinases and 3 different βarrestin related proteins have been isolated (see for references Haribabu and Snyderman, 1993). Apparently this family of G-protein coupled receptor kinases and βarrestin related proteins is involved in a general occurring regulatory mechanism for G-protein coupled receptors.

During the last few years, the genes encoding the canine, rat and human histamine H_2 receptor have been cloned (Gantz *et al.*, 1991a, b, Ruat *et al.*, 1991). Several potential phosphorylation sites appear to exist in the cytoplasmatic tail (Gantz *et al.*, 1991a, b, Ruat *et al.*, 1991). As the observed histamine-induced histamine H_2 receptor desensitization in U937 cells was found to be homologous, these sites might be possible targets for a specific receptor kinase. Moreover, recently Chuang *et al.* (1992) have reported the presence of β-adrenoceptor kinase in U937 cells, further supporting this possibility. The marked differences between the observed time course of histamine H_2 receptor desensitization in the different cellular systems may be explained by a difference in distribution of molecular entities, such as specific receptor kinases or related βarrestins.

In conclusion, we have shown that the U937 cell line expresses classical histamine H_2 receptors which can serve as a suitable model to study receptor regulation. These endogenous histamine H_2 receptors were found to be susceptible to desensitization, a mechanism which may become apparent under pathophysiological conditions or during drug therapy. Ungerer et al. (1993) have reported an increased expression of β-adrenoceptor kinase during cardiac heart failure. Considering the possible application of H_2 agonists during cardiac heart failure (Felix *et al.*, 1991) it is important to know whether the histamine H_2 receptor is also substrate for this kinase. In view of effective drug therapy detailed knowledge of the mechanism underlying

histamine H_2 receptor desensitization is required.

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Chapter 5

Two distinct pathways for histamine H_2 receptor downregulation

H_2 $Leu^{124}Ala$ receptor mutant provides evidence for a cAMP-independent action of H_2 agonists

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In the present study we have demonstrated that the rat histamine H_2 receptor density in Chinese Hamster Ovary cells ($CHOrH_2$ cells) is affected by a long-term exposure to H_2 agonists. Pretreatment of $CHOrH_2$ cells with histamine resulted in a time-dependent ($t_{1/2} \approx 7$ hrs at a concentration of 100 μM) and dose-dependent (EC_{50} = 18 nM at 24 hrs incubation) H_2 receptor downregulation measured as [^{125}I]-iodoaminopotentidine binding (44 ± 10% maximal downregulation).

Activation of the protein kinase A pathway by cholera toxin (1-100 ng/ml) or forskolin also leads to H_2 receptor downregulation. Forskolin time-dependently ($t_{1/2} \approx 7$ hrs, concentration of 10 μM) and dose-dependently (EC_{50} = 0.3 μM at 24 hrs incubation) induced H_2 receptor downregulation, whereas its inactive analogue dideoxyforskolin (1-10 μM) did not. A comparison of the time course and maximum extent of the histamine and forskolin-induced H_2 receptor downregulation suggests the involvement of cAMP in the process of H_2 receptor downregulation. These findings were further supported by the observation that both histamine and forskolin induced rapid downregulation of H_2 receptor mRNA levels, most likely caused by mRNA destabilization, thereby contributing to the observed decrease of H_2 receptor binding sites. Recently, Moro *et al.* (1993) have shown that hydrophobic amino acids in a conserved G-protein coupled receptor motif in the second intracellular loop are involved in G-protein coupling. To assess the role of cAMP in the process of agonist-induced H_2 receptor downregulation we uncoupled the H_2 receptor from the G_s protein by substitution of the $leucine^{124}$ in this G-protein coupled receptor motif by an alanine residue. The H_2 $Leu^{124}Ala$ mutant indeed showed an impaired ability of the receptor to physically and functionally couple to its G-protein as demonstrated by altered agonist-binding parameters (disappearance of high affinity binding site, no detectable GTPγS shift) and attenuated histamine-induced cAMP production (reduction of EC_{50} value and decrease of maximum response). Since the H_2 $Leu^{124}Ala$ receptor mutant was downregulated by concentrations of histamine that did not give rise to cAMP production the agonist-induced downregulation mainly concerns a cAMP-independent pathway.

Introduction

The introduction of molecular biology in the field of histamine receptor research has greatly improved the possibilities to study molecular aspects of histamine receptor proteins. In 1991 Gantz *et al.* cloned the cDNA encoding the canine histamine H_2 receptor (Gantz *et al.*, 1991b), which was followed by the cloning of both the rat and human homologues (Gantz *et al.*, 1991a, Ruat *et al.*, 1991). The deduced amino acid sequence of the H_2 receptor proteins reveals the existence of seven putative transmembrane domains, indicating that this receptor is a member of the large family of G-protein coupled receptors (GPCRs). This family of receptors is known to be readily subjected to regulatory processes in order to control receptor signalling, thus maintaining biological equilibria within the cell (Lohse, 1993). Short-term exposure of

receptors to high concentrations of agonists is often followed by a decrease in cellular responsiveness, called desensitization (Hausdorff *et al.*, 1991). Long-term exposure, on the other hand, results in the reduction of receptor number (Collins *et al.*, 1991) and is referred to as receptor downregulation. Since the histamine H_2 receptor is a member of this family of GPCRs, it is not surprising that this receptor is also susceptible to such regulatory mechanisms.

Recently, we have shown that in human U937 cells the endogenously expressed histamine H_2 receptors are indeed rapidly desensitized when exposed to histamine (Smit *et al.*, 1994). Similar observations have been reported in other cellular systems (Arima *et al.*, 1993, Fukushima *et al.*, 1994). Yet, so far no detailed information is available on long-term desensitization of the histamine H_2 receptor such as receptor downregulation. Such processes may become apparent under several pathophysiological conditions (e.g. asthmatic attack or allergic reactions in general), during which histamine is released in large quantities, but might also occur under normal physiological conditions. Recently, Diaz *et al.* (1994) suggested e.g. that *in vivo* receptor downregulation might explain the inverse relationship between H_2 receptor expression and the localization of histamine-synthesizing cells in the rodent gastric wall. The regulation of H_2 receptor expression has further gained interest due to the potential therapeutical application of H_2 receptor agonists in patients sufferering from congestive heart failure (Felix *et al.*, 1991). Moreover, it has been reported recently that striatal histamine H_2 receptor density in brain tissue of patients suffering from Huntington's Chorea is markedly reduced (Martinez-Mir *et al.*, 1993).

Investigation of the regulation of H_2 receptor expression has so far been hampered by the availability of suitable model systems. Cellular systems (Arima *et al.*, 1993, Fukushima *et al.*, 1994, Holden *et al.*, 1987, Smit *et al.*, 1994) have been used to investigate second messenger responses coupled to the histamine H_2 receptor stimulation, but the used systems such as e.g. U937 cells do not express a sufficiently high density of H_2 receptors to permit radioligand binding studies, which are essential for the investigation of long-term regulatory mechanisms (Smit *et al.*, 1994). Following the recent cloning of cDNAs or genes encoding histamine H_2 receptors, cell lines expressing considerable amounts of histamine H_2 receptors can be obtained (Leurs *et al.*, 1994, Traiffort *et al.*, 1992). Additionally, the availability of the H_2 receptor gene allows the construction of receptor mutants, which can provide mechanistic insights in phenomena like receptor downregulation.

In the present study we have examined the effects of long-term exposure of the rat histamine H_2 receptor stably expressed in Chinese Hamster Ovary (CHO) cells ($CHOrH_2$) (Traiffort *et al.*, 1992) to H_2 agonists and cAMP mobilizing agents with regard to H_2 receptor protein expression and H_2 receptor mRNA levels. In order to get more insight in the mechanisms underlying H_2 receptor regulation, we constructed by means of site-directed mutagenesis a H_2 receptor mutant, in which leucine^{124} in the second intracellular loop was substituted by an alanine. This H_2 Leu^{124}Ala receptor mutant was as expected partially

uncoupled from its G-protein and was therefore an excellent tool to investigate the existence of possible cAMP-dependent and independent pathways in the process of agonist-induced H_2 receptor downregulation.

Materials and methods

Cell culture CHO cells expressing the rat histamine H_2 receptor ($CHOrH_2$) (Traiffort *et al.*, 1992) and the mutated $H_2Leu^{124}Ala$ receptor ($CHOrH_2Leu^{124}Ala$) were grown at 37°C in a humified atmosphere with 5% CO_2 in Dulbeco's Modified Eagle's Medium (DMEM), containing 10% (vol/vol) dialyzed foetal calf serum supplemented with 2 mM L-glutamine, MEM amino acids, 50 IU/ml penicillin and 50 µg/ml streptomycin.

Site-directed mutagenesis The H_2 receptor mutant, in which leucine^{124} was substituted by alanine ($Leu^{124}Ala$) (Fig. 5), was constructed by means of the Polymerase Chain Reaction. The oligonucleotides S1 (5'-GGGAAGCTTGGCCCCAGAATGGAGCCCAATG GCACAGT), corresponding to nucleotides -9 to 21 (Ruat *et al.*, 1991) and a *Hind*III site (underlined), and AS1 (5'-GGGGGTACCGCGCTGGGT-CCGTGACAGCGCAGTAGTTG TTCAAGCTGAT CAT), corresponding to nucleotides 358 to 383 of the complementary strand (Ruat *et al.*, 1991) containing a *Kpn*I site (underlined) with two nucleotide changes, were synthesized on an Applied Biosystems DNA synthesizer (model 381A). Using 100 ng $pSVrH_2$ (Traiffort *et al.*, 1992) as a template, 0.4 µM S1, 0.4 µM AS2, 40 µM dNTP's and 2.5 U Amplitaq according to the manufacturing protocol (Perkin Elmer) a 392 bases DNA fragment of the H_2 $Leu^{124}Ala$ receptor mutant was amplified in 100 µl using 30 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1.5 min and a final extension at 72°C for 10 mins. The obtained PCR product was gel-purified, and restricted with *Hind*III/*Kpn*I (Boehringer). This fragment was cloned into the plasmid pSP73 (Promega) containing the wild-type rH_2 receptor, which was restricted with *Kpn*I and *Hind*III. Thereafter, the PCR amplified sequence was verified using the dideoxy-chain termination method with the Sequenase kit (USB). Subsequently, the coding sequence of the mutated $H_2Leu^{124}Ala$ receptor was subcloned into the eukaryotic pSV expression vector. CHO cells, deficient in dihydrofolate reductase, were stably transfected with 15 µg of $pSVrH_2Leu^{124}Ala$ using Transfectam (Promega).

Membrane preparation $CHOrH_2$ and $CHOrH_2Leu^{124}Ala$ cells were washed three times with cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and harvested with a cell scraper and recovered by a 10 min centrifugation at 500g. Cells were homogenized in ice-cold 50 mM Na_2/K-phosphate buffer (pH = 7.4) with a Polytron homogenizer (5 secs, maximal speed) and used for radioligand binding studies. Protein concentrations were determined according to Bradford using bovine serum albumin as a standard (Bradford, 1976).

Histamine H_2 receptor binding The radiolabelled H_2 receptor antagonist [^{125}I]-iodoaminopotentidine ([^{125}I]-APT) was synthesized as described previously (Leurs *et al.*, 1994). Triplicate assays were performed in polyethylene tubes in 50 mM Na_2/K phosphate buffer containing gelatine (0.1%) to prevent adsorption of the radioligand. In saturation studies increasing concentrations of [^{125}I]-APT were incubated with 5-10 µg of membrane proteins in the absence or presence of 1 µM tiotidine in a total volume of 400 µl. After 90 mins at 30°C the incubations were stopped by rapid dilution with 3 ml ice-cold 20 mM Na_2/K phosphate buffer (pH = 7.4) supplemented with 0.1% bovine serum albumin. The bound radioactivity was subsequently separated by filtration with a Brandel cell harvester (Semat, United Kingdom) through Whatman GF/B glass fibre filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 ml buffer and radioactivity retained on the filters was counted with a LKB-γ-counter at an efficiency of 63%. The binding data were evaluated by use of LIGAND, a non-linear, weighted, least squares curve-fitting procedure (Munson and Rodbard, 1980). Changes in H_2 receptor density were denoted as a percentage downregulation compared to non-treated control cells. During the 24 hrs incubation of cells with various histamine ligands or other compounds, cells were maintained in medium without foetal calf serum.

Cyclic AMP production $CHOrH_2$ and $CHOrH_2Leu^{124}Ala$ cells were seeded in 24-well plates and cultured overnight in culture medium. Cells were washed twice with DMEM, supplemented with 50 mM HEPES (pH = 7.4 at 37°C) and preincubated for 30 mins at 37°C. Thereafter, the medium was aspirated, appropriate drugs in DMEM/HEPES supplemented with 300 µM of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) were added and the cells were incubated for 10 mins at 37°C. The reaction was stopped by the rapid aspiration of the culture medium and the addition of 200 µl of 0.1 N cold HCl. The cells were kept on ice and disrupted by

sonification (5 secs, 50 Watt, Labsonic 1510, Braun-Melsungen, Germany). The resulting homogenate was frozen at -20°C or directly neutralized with 1 N NaOH and assayed for the presence of cAMP.

Cyclic AMP assay The amount of cAMP in the $CHOrH_2$ and $CHOrH_2Leu^{124}Ala$ cells was determined according to Nordstedt and Fredholm (Nordstedt and Fredholm, 1990), with some minor modifications. Briefly, a protein kinase A containing fraction was isolated from bovine adrenal glands. Adrenal cortex was homogenized in 10 volumes 100 mM Tris/HCl, 250 mM NaCl, 10 mM EDTA, 0.25 M sucrose and 0.1% 2-mercaptoethanol (pH = 7.4 at 4°C, buffer A) using an Omni-Sorval mixer (30 secs, maximal speed) and a Polytron homogenizer (10 secs, maximal speed). The homogenate was centrifuged for 60 mins at 30,000g at 4°C. The supernatant, containing protein kinase A, was carefully recovered and frozen in 1 ml aliquots at -80°C. Before use, the binding protein was diluted five-fold in ice-cold buffer A without sucrose and 2-mercaptoethanol and kept on ice. Subsequently, 200 µl of the binding protein was mixed with 50-100 µl of the CHO homogenate or cAMP standards and 30.000 dpm [^{3}H]-cAMP. After incubation for 150 mins at 4°C the mixture was rapidly diluted with 3 ml ice-cold 50 mM Tris/HCl (pH = 7.4 at 4°C) and filtered through Whatman GF/B filters using a Brandel cell-harvester (Semat, UK). The radioactivity retained on the filters was measured by liquid scintillation counting.

RNA slot blot analysis RNA was analyzed by means of mRNA slot blot assay as described by Zhang *et al.* (1994), with minor modifications. Briefly, RNA was isolated according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987), using Trizol reagent® (GIBCO BRL), and RNA was slot blotted on a nitrocellulose filter (GeneScreen plus, NEN) and prehybridized for 2 hrs at 65°C in 7% SDS, 0.5 M Na_2HPO_4, 1 mM EDTA (pH 7.2). Thereafter, filters were hybridized overnight with a radioactive labelled 48-mer antisense rat H_2 receptor oligonucleotide (5'-GATGGTGGCTGCCTTCCAGGAGCTGATGTG-GTTGATCCGTTTGGCCTG-3', corresponding to nucleotides 631 to 678) or antisense rat β-actin oligonucleotide (5'-CTCCTGCTTGCTGATCCACATCTGCTGGAAGGTGGACAGTGAGGCCAG-3', corresponding to nucleotides 3040 to 3087) at 65°C in 7% SDS, 0.5 M Na_2PO_4 and 1 mM EDTA (pH 7.2). The rat H_2 receptor oligonucleotide (1.5 pmol) was [^{32}P]-labelled by 3'-end tailing using 16 pmol of [α-^{32}P]-dATP (3000 Ci/mmol; Amersham) and 1 unit of terminal deoxynucleotidyl transferase (Boehringer) for 20 min at 37°C. The β-actin oligonucleotide (5 pmol) was labelled using 10 pmol of [γ-^{32}P]-dATP (3000 Ci/mmol; Amersham) and 4 units of polynucleotide kinase (Boehringer) for 30 mins at 37°C. The blots were washed twice for 5 mins at room temperature in 2xSSC (0.3 M NaCl, 0.03 M $Na_3C_6H_5O_7.2H_2O$) supplemented with 0.1% SDS, which was followed by two 45 mins washes at 65°C with 2xSSC supplemented with 0.1% SDS. The blots were exposed to a phosphorscreen (Molecular Dynamics) and signals were quantified with a phosphorimager 425 (Molecular Dynamics) using the computer program ImageQuant (Molecular Dynamics). H_2 receptor mRNA levels were expressed as the ratio of the values of the H_2 receptor mRNA signals and the corresponding β-actin signals.

Analysis of H_2 receptor mRNA stability H_2 receptor mRNA levels were determined after incubation of the $CHOrH_2$ cells with actinomycine D to block transcription as described previously (Rodgers *et al.*, 1985). Cells were preincubated with or without 100 µM histamine or with 10 µM forskolin for 1 hr in DMEM. Thereafter, actinomycin D (10 µg/ml) was added. Cells were harvested from 0 to 90 mins after addition of actinomycin D. Total RNA was extracted at each time point and H_2 receptor mRNA was quantified by means of the mRNA slot blot assay as described above.

Chemicals Histamine dihydrochloride, isobutylmethylxanthine (IBMX), cyclic AMP (cAMP), forskolin, 1,9 dideoxyforskolin, cholera toxin and GTPγS were obtained from Sigma Chemical Company (USA). Actinomycin D was purchased from Boehringer (Mannheim, Germany). [2,8-^{3}H]-cAMP (40 Ci/mmol) was obtained from Amersham. Dimaprit dihydrobromide, homo- and nordimaprit dihydrochloride, amthamine dihydrobromide, amselamine dihydrobromide and aminopotentidine were taken from laboratory stock. Gifts of cimetidine (SmithKline Beecham, United Kingdom), ranitidine dihydrochloride (Glaxo, United Kingdom), tiotidine (Imperial Chemical Industries, United Kingdom), CHO cells expressing the rat H_2 receptor and $pSVrH_2$ vector (Dr J.C. Schwartz) are greatly acknowledged.

Statistical analysis All data shown are expressed as mean ± standard error (mean ± s.e.mean) of at least three independent experiments. Statistical analysis was carried out by Student's *t*-test. P-values < 0.05 were considered to indicate a significant difference.

Results

Histamine-induced H_2 receptor downregulation

Exposure of $CHOrH_2$ cells (Traiffort *et al.*, 1992) to 100 μM of histamine for prolonged periods of time resulted in a time-dependent decrease of [^{125}I]-iodoaminopotentidine ([^{125}I]-APT) binding (Fig. 1A). Maximum reduction of [^{125}I]-APT binding was observed after 16 hrs incubation of cells with 100 μM of histamine. Under this condition, histamine induced 44 ± 10% ($p < 0.05$) reduction of [^{125}I]-APT binding. Half maximum reduction of the [^{125}I]-APT binding was recorded at an incubation period of approximately 7 hrs. A 24 hrs incubation of $CHOrH_2$ cells with increasing concentrations of histamine led to a dose-dependent reduction of [^{125}I]-APT binding (EC_{50} value = 18 ± 6 nM, mean ± s.e.mean, n = 7) (Fig. 1B). The observed reduction of [^{125}I]-APT binding was not reflected by a change in affinity of [^{125}I]-APT for the H_2 receptor, as its dissociation constant (K_d), determined by means of saturation studies, remained unaffected in $CHOrH_2$ cells incubated with 100 μM histamine for 24 hrs (Table 1). Exposure of $CHOrH_2$ cells to histamine resulted only in a marked decrease of the total number of [^{125}I]-APT binding sites (B_{max}) (Table 1).

The recently described selective H_2 receptor agonists amselamine and amthamine (Eriks *et al.*, 1992, Van der Goot *et al.*, 1994) induced cAMP production in $CHOrH_2$ cells, with EC_{50} values lower and maximal responses comparable to histamine (Table 2). Long-term exposure (24 hrs) of $CHOrH_2$ cells to 100 μM of amselamine or 100 μM of amthamine resulted in a decrease of [^{125}I]-APT binding of 41 ± 1% and 56 ± 6% respectively. As shown in Table 2, 24 hrs incubation of $CHOrH_2$ cells with 100 μM of dimaprit, which generates cAMP slightly less efficient compared to histamine induced 33 ± 7% decrease of [^{125}I]-APT binding sites. Dimaprit's structural analogues, homodimaprit and nordimaprit showed strongly reduced capacities to generate cAMP with EC_{50} values of 1.4 ± 0.9 μM and higher than 10

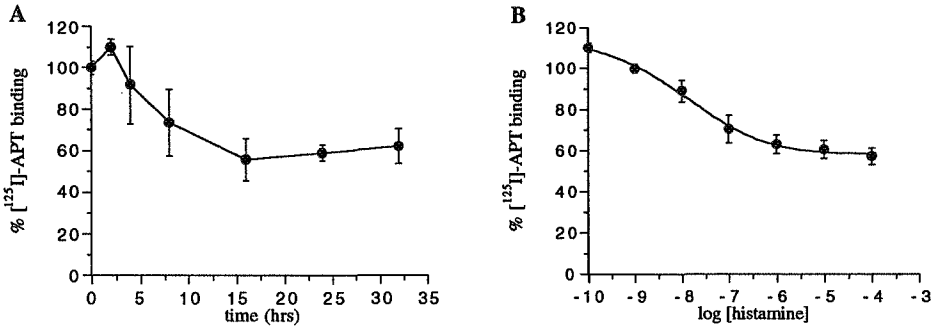

Fig. 1. Time- and dose-dependent decrease of [^{125}I]-APT binding in $CHOrH_2$ cells by histamine. A, $CHOrH_2$ cells were incubated with 100 μM of histamine for the indicated times and [^{125}I]-APT binding in membranes was measured. The [^{125}I]-APT binding is expressed as a percentage of [^{125}I]-APT binding measured in non-treated cells. The data shown represent the mean ± s.e.mean of 4 independent experiments. B, Dose-dependent decrease of [^{125}I]-APT binding induced by histamine. $CHOrH_2$ cells were exposed to various concentrations of histamine for 24 hrs. The data represent the mean ± s.e.mean of 7 independent experiments.

μM respectively (Table 2). The reduced ability of these dimaprit analogues to induce a cAMP response was paralleled by a lack of H_2 receptor downregulation after 24 hrs incubation of CHOrH_2 cells with 100 μM of the analogues (Table 2).

Table 1 Characteristics of the [^{125}I]-APT binding to the rat histamine H_2 receptor expressed in CHO cells pretreated for 24 hrs with or without histamine or forskolin

	[^{125}I]-APT binding	
pretreatment 24 hrs	K_d (nM)	B_{max} (fmol/mg protein)
control	0.43 ± 0.06	975 ± 12
histamine (100 μM)	0.42 ± 0.05	468 ± 129 *
forskolin (10 μM)	0.29 ± 0.03 *	324 ± 59 *

The dissociation constant (K_d) and maximum number of binding sites (B_{max}) were determined using non-linear fitting according to a one-site binding mode. The data shown represent the mean ± s.e.mean of 3 independent experiments. The asterisk indicates a significant difference ($p < 0.05$) from control, represented by untreated cells

Table 2 EC_{50}-values and the percentage of maximally-induced production (E_{max}) of different H_2 agonists for production of cAMP and the effect of 24 hrs incubation of these compounds (100 μM) on [^{125}I]-APT binding in CHOrH_2 cells

	cAMP response		
	EC_{50}	% E_{max}	% downregulation
histamine	66.1 ± 29.1 nM	100 ± 4	41 ± 6 *
amselamine	14.5 ± 5.9 nM	88 ± 10	41 ± 1 *
amthamine	5.3 ± 0.7 nM	89 ± 6	56 ± 6 *
dimaprit	0.20 ± 0.07 μM	122 ± 8	33 ± 7 *
nordimaprit	> 10 μM	70 ± 3#	7 ± 9
homodimaprit	1.35 ± 0.85 μM	38 ± 1	11 ± 5

Data of the E_{max} values are shown as a percentage of the histamine (100 μM)-induced response. The effect of 24 hrs of incubation of CHOrH_2 cells with the different compounds on [^{125}I]-APT binding is expressed as the percentage of H_2 receptor downregulation. The data shown represent the mean ± s.e.mean of 4 to 8 independent experiments. The asterisk indicates a significant difference ($p < 0.05$) from control, represented by non-treated cells. #; The E_{max} value of nordimaprit was determined using a concentration of 1 mM.

Role of cAMP in the process of H_2 receptor downregulation

Forskolin, which directly activates adenylyl cyclase, dose-dependently induced the formation of cAMP in $CHOrH_2$ cells (Fig. 2A). Prolonged exposure (incubation periods ranging from 4 to 32 hrs) of $CHOrH_2$ cells with 10 μM of forskolin led to a marked reduction of 58 ± 2% of [^{125}I]-APT binding (Fig. 2B). Again, no major change in affinity of [^{125}I]-APT for the H_2 receptor was apparent, only a decrease in B_{max} was observed when $CHOrH_2$ cells were incubated for 24 hrs with 10 μM of forskolin (Table 1). Maximum and half-maximum down-

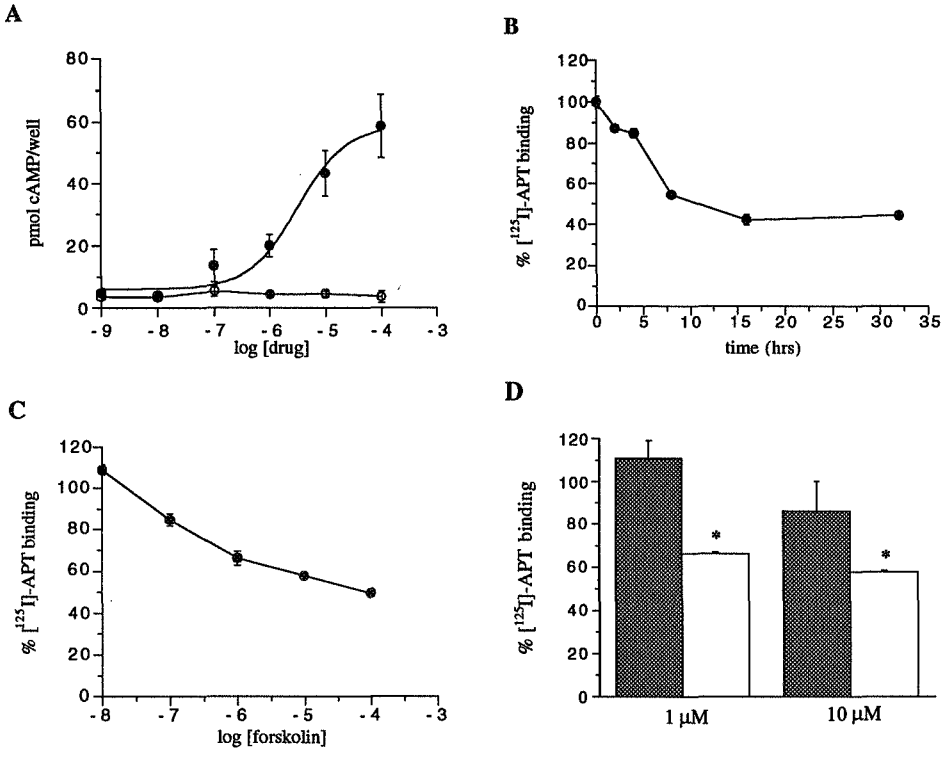

Fig. 2. Forskolin- and 1,9-dideoxyforskolin-induced cAMP production and decrease of [^{125}I]-APT binding in $CHOrH_2$ cells. A, Dose-dependent increase of the cAMP production by forskolin (filled circles) and 1,9-dideoxyforskolin (open circles). $CHOrH_2$ cells were incubated with the indicated drugs with increasing concentrations for 10 mins at 37°C in DMEM in the presence of 300 μM of IBMX and 25 mM of Hepes, pH 7.4. Data represent the mean ± s.e.mean of 6 independent experiments. B, Forskolin-induced decrease of [^{125}I]-APT binding in $CHOrH_2$ cells. $CHOrH_2$ cells were incubated with 10 μM of forskolin for the indicated times and [^{125}I]-APT binding was measured. The [^{125}I]-APT binding is expressed as a percentage of [^{125}I]-APT binding measured in non-treated cells. C, Dose-dependent decrease of [^{125}I]-APT binding in $CHOrH_2$ cell membranes induced by forskolin. $CHOrH_2$ cells were exposed to various concentrations of forskolin for 24 hrs. The H_2 receptor density was determined as described. D, Effect of 1,9 dideoxyforskolin and forskolin on [^{125}I]-APT binding. $CHOrH_2$ cells were exposed to 1 and 10 μM of 1,9 dideoxy forskolin (filled bars) and forskolin (open bars) respectively for 24 hrs and examined for [^{125}I]-APT binding. The asterisk indicates a significant difference ($p < 0.05$) from control, represented by the [^{125}I]-APT binding measured in untreated cells. Data from B, C and D were calculated as mean ± s.e.mean from 4 independent experiments.

regulation was recorded after 16 hrs and approximately 7 hrs of incubation of $CHOrH_2$ cells with 10 μM forskolin respectively (Fig. 2B). The H_2 receptor binding sites appeared to be dose-dependently downregulated by increasing concentrations of forskolin, with an EC_{50} value of 0.3 ± 0.06 μM (mean ± s.e.mean, n = 4) (Fig. 2C). Concentrations up to 10 μM of the inactive analogue 1,9-dideoxy-forskolin, which does not generate cAMP in $CHOrH_2$ cells (Fig. 2A), did not attenuate the H_2 receptor density after 24 hrs of pretreatment (Fig. 2D).

Cholera toxin (CTX), which irreversibly activates the G_s protein, thereby generating cAMP, also induced a dose-dependent decrease of [^{125}I]-APT binding sites when incubated for 24 hrs (EC_{50} = 32 ± 1 ng/ml, mean ± s.e.mean, n=4, Fig. 3). CTX pretreatment of $CHOrH_2$ cells resulted in a maximum downregulation of H_2 receptors of 46 ± 3%. Finally, exposure of $CHOrH_2$ cells for 24 hrs to 300 μM of IBMX, a cAMP phosphodiesterase inhibitor, also resulted in an attenuation of [^{125}I]-APT binding (26 ± 7% H_2 receptor downregulation, n = 4, mean ± s.e.mean, $p < 0.05$).

H_2 receptor mRNA levels and stability in control, histamine-treated and forskolin-treated $CHOrH_2$ cells

Exposure of $CHOrH_2$ cells to 100 μM of histamine for increasing periods of time resulted in a rapid transient decrease of H_2 receptor mRNA (maximal reduction of 71 ± 4%, mean ± s.e.mean, n = 4) (Fig. 4). This effect was at its peak after 4 hrs of incubation of cells with histamine (100 μM), while the amount of H_2 receptor mRNA returned to approximately 50% of control after 12 hrs of histamine-treatment. Long-term incubation of $CHOrH_2$ cells with 10 μM of forskolin also induced a time-dependent transient decrease (maximal reduction: 75 ± 7%

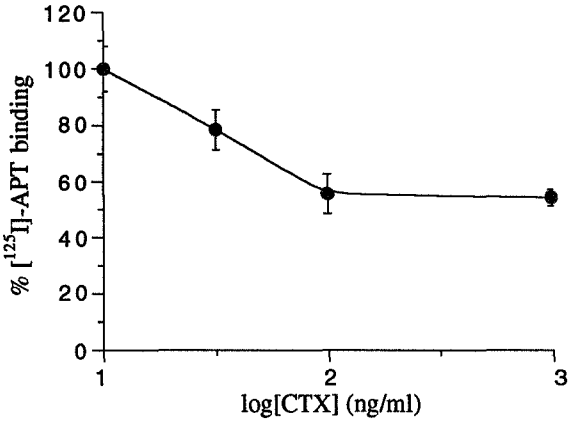

Fig. 3. Effect of long-term treatment with CTX on [^{125}I]-APT binding in $CHOrH_2$ cells. The $CHOrH_2$ cells were treated with increasing concentrations of CTX for 24 hrs. [^{125}I]-APT binding is expressed as a percentage of [^{125}I]-APT binding measured in non-treated cells studied under the same conditions and was measured as described earlier. The data shown represent the mean ± s.e.mean of 4 independent experiments.

mean ± s.e.mean, n = 4) of H_2 receptor mRNA to levels similar to those observed after histamine-treatment (Fig. 4).

To study the role of mRNA stability $CHOrH_2$ cells were incubated for 1 hr in the absence or presence of histamine (100 μM) or forskolin (10 μM), whereafter actinomycin D (10 μg/ml) was added to block mRNA transcription. Cells were collected at different time intervals ranging from 0 to 90 mins after addition of actinomycin D and were analyzed for H_2 receptor mRNA content. The H_2 receptor mRNA in non-treated cells was hardly affected during the 90 mins of incubation with actinomycin D (Inset Fig. 4). Incubation of cells with 100 μM of histamine, however, resulted in a significant breakdown of H_2 receptor mRNA levels (Inset Fig. 4). Similar results were obtained after forskolin treatment (Inset Fig. 4).

As can be seen in Fig. 4, 4 hrs of incubation of cells with 100 μM of histamine resulted in a marked downregulation (71 ± 4%) of the H_2 receptor mRNA, whereas after 4 hrs of incubation of $CHOrH_2$ cells and direct measurement of [^{125}I]-APT binding no significant downregulation of H_2 receptors was observed (Fig. 1A).Yet, when $CHOrH_2$ cells were incubated with 100 μM of histamine for 4 hrs, extensively washed and further incubated in serum-free medium without histamine for another 20 hrs, a significant reduction of 60 ± 1% (mean ± s.e.mean, n = 4) of the H_2 receptor binding sites was observed.

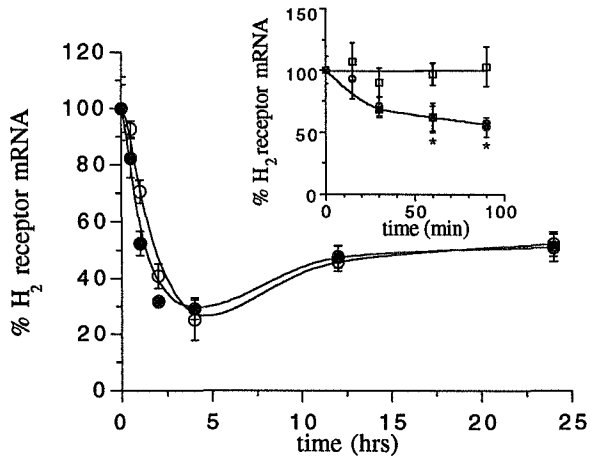

Fig. 4. Histamine- and forskolin-induced modulation of H_2 receptor mRNA levels. $CHOrH_2$ cells were incubated for the indicated times with 100 μM of histamine (filled circles) or 10 μM of forskolin (open circles) and mRNA quantified by means of a RNA slot blot assay as described under "Experimental procedures". The results are the mean ± s.e.mean of two separate experiments, performed in duplicate. Inset, Effect of histamine-treatment and forskolin-treatment on H_2 receptor mRNA stability. $CHOrH_2$ cells were incubated with (open circles) or without (open squares) 100 μM of histamine or with 10 μM of forskolin (filled squares) for 1 hr, before actinomycin D (10 μg/ml) was added. Cells were harvested at 0, 15, 30, 60 and 90 mins after the addition of actinomycin D. The data are the mean ± s.e.mean of three experiments, each performed in duplicate. The asterisk indicates a significant difference ($p < 0.05$) from control, represented by untreated cells.

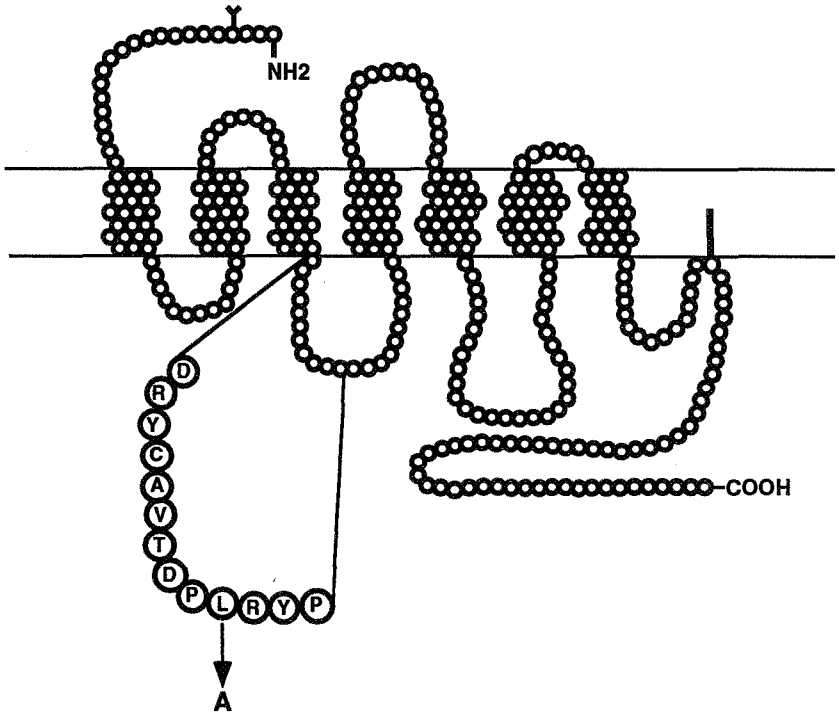

Fig. 5. Schematic representation of the rat histamine H_2 receptor. The leucine (L) at position 124, located in the second intracellular loop, was mutated to an alanine (A) by site-directed mutagenesis as described under Materials and methods.

Functional analysis of the Leu^{124}Ala mutation of the rat histamine H_2 receptor

Using the polymerase chain reaction the leucine^{124} in the second intracellular loop of the rat histamine H_2 receptor (Fig. 5) was mutated into an alanine residue. Transfection of the H_2Leu^{124}Ala receptor cDNA into CHO cells, resulted in the formation of several clonal cell lines expressing [^{125}I]-APT binding sites. A clonal cell line expressing amounts of [^{125}I]-APT binding comparable to those of the CHOrH_2 cells was chosen for further analysis and referred to as CHOrH_2Leu^{124}Ala (CHOrH_2 cells: 975 ± 12 fmol/mg of protein, CHOrH_2Leu^{124}Ala cells: 980 ± 7 fmol/mg of protein, mean ± s.e.mean, n = 3). There were no major differences in the binding of the H_2 antagonists to the wild-type receptor or the mutated receptor. The affinity of [^{125}I]-APT for the mutated receptor was hardly affected (K_d of [^{125}I]-APT in CHOrH_2 cells: 0.43 ± 0.06 nM, in CHOrH_2Leu^{124}Ala cells: 0.61 ± 0.03 nM, mean ± s.e.mean, n = 3). Moreover, cimetidine and ranitidine had similar K_i values for both receptors (Table 3). In contrast, the introduced Leu^{124}Ala mutation significantly affected the agonist binding characteristics. In CHOrH_2 cells, histamine displacement curves were shallow and could be analysed best by a two site model (Fig. 6, Table 3). The addition of 10

μM GTPγS resulted in a steepening and a right-ward shift of the histamine displacement curve, which could be analysed best by a single site model with a K_i value of 0.18 ± 0.02 mM (Fig. 6). In CHOrH_2Leu^{124}Ala cells, however, the displacement curve of histamine was analysed best by a single site model, leading to a K_i value (0.21 ± 0.02 mM) that corresponded to the low affinity site of the wild-type receptor (Fig. 6, Table 3). The addition of 10 μM of GTPγS did not result in a right-ward shift of the displacement curve of histamine (Fig. 6, Table 3).

Moreover, the Leu^{124}Ala mutation also affected the ability of histamine to induce the formation of cAMP in CHOrH_2Leu^{124}Ala cells (fig. 7A). The EC_{50} value of the histamine-induced cAMP response in CHOrH_2Leu^{124}Ala cells was approximately 162-fold higher (11 ± 3 μM, mean ± s.e.mean, n = 7) than the observed EC_{50} value of the histamine-induced cAMP response in CHOrH_2 cells (66 ± 29 nM, mean ± s.e.mean, n = 6) measured under the same conditions. The maximum histamine-induced reponse was also found to be affected in CHOrH_2Leu^{124}Ala cells (E_{max} in CHOrH_2 cells: 40 ± 4 pmol/well, E_{max} in CHOrH_2Leu^{124}Ala cells: 18 ± 1 pmol/well).

Histamine-induced downregulation of rat H_2 Leu^{124}Ala receptors

Long-term exposure (24 hrs) of CHOrH_2Leu^{124}Ala cells to increasing concentrations of histamine resulted in a dose-dependent reduction of [^{125}I]-APT binding sites (Fig. 7B). Whereas in CHOrH_2 cells an EC_{50} of 18 ± 6 nM (mean ± s.e.mean, n = 7) was observed for histamine, in CHOrH_2Leu^{124}Ala cells histamine induced downregulation with an EC_{50} value of 288 ± 89 nM (mean ± s.e.mean, n = 4). Comparing the histamine-induced cAMP production and H_2 Leu^{124}Ala receptor downregulation (Fig. 7B), a discrepancy in dose-relationships is observed. Almost 40-fold higher concentrations of histamine are required to induce cAMP production, compared to receptor downregulation. Pretreatment of CHOrH_2Leu^{124}Ala cells for 24 hrs with 1 μM of histamine resulted in a significant degree of H_2 receptor downregulation (51 ± 2%, mean ± s.e.mean, n = 4), whereas no significant cAMP production was observed after 10 mins of incubation (Fig 7B). Also after 24 hrs of incubation of CHOrH_2Leu^{124}Ala cells with 1 μM of histamine, no significant increase in cAMP was observed (M.J. Smit, unpublished observations). Moreover, even at 0.1 μM of histamine significant H_2 receptor downregulation was observed. The mRNA levels of the H_2Leu^{124}Ala mutant were not decreased upon 24 hrs incubation with 1 μM of histamine (101 ± 19% vs non-treated cells, mean ± s.e.mean, n = 4). A 24 hrs incubation of CHOrH_2 cells with 1 μM of histamine, on the other hand, resulted in a significant decrease of H_2 receptor mRNA levels (45 ± 2% vs non-treated cells , mean ± s.e.mean, n=4).

In the CHOrH_2Leu^{124}Ala cells the maximum histamine-induced downregulation was more pronounced (68 ± 4%, mean ± s.e.mean, n = 4) than was observed for the CHOrH_2 cells (43 ± 4%, mean ± s.e.mean, n = 7). The forskolin (10 μM)-induced H_2 receptor downregulation was also found to be more pronounced in the CHOrH_2Leu^{124}Ala cells (67 ± 1%, mean ± s.e.mean, n = 3) than in CHOrH_2 cells (58 ± 2%, mean ± s.e.mean, n = 4).

Table 3 Pharmacological characterization of [^{125}I]-APT binding to CHOrH$_2$ and CHOrH$_2$Leu^{124}Ala cell membranes

	K_i	
compounds	CHOrH$_2$	CHOrH$_2$Leu^{124}Ala
cimetidine	804 ± 53 nM	766 ± 73 nM
ranitidine	232 ± 45 nM	173 ± 20 nM
histamine	$K_{i,\ high}$ = 3.5 ± 1.0 µM (39 ± 7%) $K_{i,\ low}$ = 0.24 ± 0.04 mM (61 ± 7%)	- 0.21 ± 0.02 mM (100%)
+ 10 µM GTPγS	0.18 ± 0.02 mM (100%)	0.23 ± 0.03 mM (100%)

Membranes of CHOrH$_2$ and CHOrH$_2$Leu^{124}Ala cells were incubated with 0.3 nM [^{125}I]-APT in the presence of the indicated drugs at increasing concentrations. K_i values were obtained from the respective IC_{50} values. Data shown are mean ± s.e.mean from at least three independent experiments. Values in parentheses indicate the relative densities of the high/low affinity binding site.

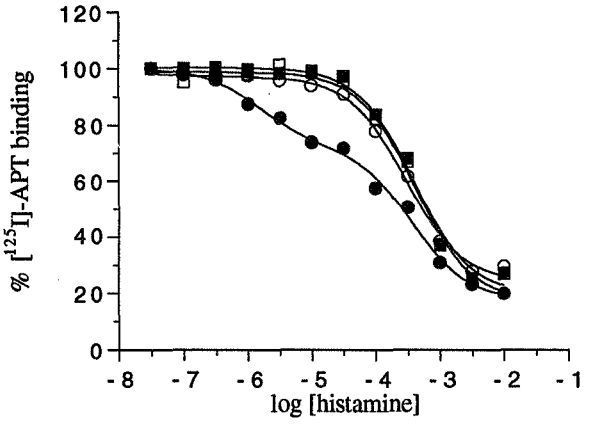

Fig. 6. Binding of histamine to the wild-type and H$_2$Leu^{124}Ala receptor. Displacement of binding of 0.3 nM of [^{125}I]-APT by increasing concentrations of histamine in the presence (open symbols) and absence (filled symbols) of 10 µM of GTPγS in CHOrH$_2$ cells (circles) and CHrH$_2$Leu^{124}Ala cells (squares). Mean values of triplicate determinations of a typical experiment out of at least three are shown.

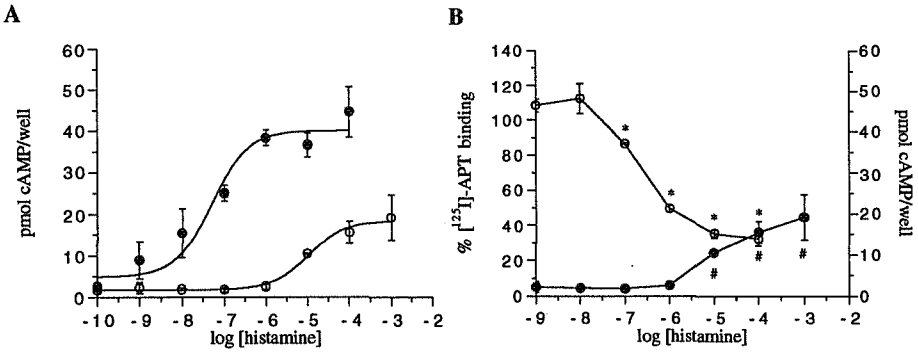

Fig. 7. Effects of $Leu^{124}Ala$ mutation on histamine-induced cAMP production and downregulation. A, Dose-dependent increase of the cAMP production by histamine in $CHOrH_2$ cells (filled circles) and $CHOrH_2Leu^{124}Ala$ cells (open circles). Cells were incubated with increasing concentrations of histamine for 10 mins at 37°C in DMEM in the presence of 300 μM of IBMX and 25 mM of Hepes, pH 7.4. The data shown represent the mean ± s.e.mean for respectively, 6 and 7 independent experiments. B, Effects of $Leu^{124}Ala$ mutation on histamine-induced H_2 receptor downregulation (open circles) and cAMP production (filled circles, see also above). $CHOrH_2Leu$ cells were exposed to increasing concentrations of histamine for 24 hrs and [^{125}I]-APT binding in membranes was measured. The [^{125}I]-APT binding is expressed as a percentage of [^{125}I]-APT binding measured in non-treated cells. The data shown represent the mean ± s.e.mean of 4 experiments. The asterisk and # indicate a significant difference ($p < 0.05$) from control, represented by untreated cells and basal cAMP levels respectively.

Discussion

So far studies addressing regulation of the histamine H_2 receptor function have focused in particular on rapid agonist-induced desensitization. These reports show that the histamine H_2 receptor is rapidly desensitized upon agonist exposure (Arima *et al.*, 1993, Fukushima *et al.*, 1994, Smit *et al.*, 1994). In contrast, no information is available on molecular mechanisms operating after prolonged periods of time (hours) of agonist stimulation.

In the present study we have demonstrated that the rat histamine H_2 receptor density in CHO cells is reduced about 50% by long-term exposure to histamine or selective H_2 agonists. Long-term treatment of $CHOrH_2$ cells with histamine resulted in a time-dependent ($t_{1/2} \approx 7$ hrs at a concentration of 100 μM) and dose-dependent (EC_{50} = 18 nM at 24 hrs of incubation) decrease in the number of H_2 receptor binding sites. Yet, incubation of $CHOrH_2$ cells with homo- and nordimaprit, two side chain homologues of the H_2 agonist dimaprit with weak H_2 agonistic activity ((Sterk *et al.*, 1987, present study), did not significantly reduce the number of H_2 receptors. These findings show that the observed H_2 agonist-induced downregulation is a H_2 receptor-mediated process.

As was found for the β_2-adrenergic receptor (β_2AR) (Bouvier *et al.*, 1989), a cAMP-dependent pathway can also regulate the H_2 receptor density. Forskolin, generating cAMP upon addition, time-dependently ($t_{1/2} \approx 7$ hrs at a concentration of 10 μM) and dose-dependently (EC_{50} = 0.3 μM at 24 hrs of incubation) induced H_2 receptor downregulation. CTX and IBMX, agents that also elevate intracellular levels of cAMP in $CHOrH_2$ cells,

induced downregulation of the H_2 receptor as well. Thus, the H_2 receptor does not need to be stimulated by an agonist in order to be downregulated. This mechanism might be involved in heterologous H_2 receptor downregulation as previously shown for other GPCRs (see for references Collins, 1993, Lohse, 1993). The time course of the forskolin-induced decrease of H_2 receptor number in CHOrH_2 cells parallels the time-dependent decrease of H_2 receptors induced by histamine. For both histamine and forskolin, half-maximum H_2 receptor downregulation is reached after approximately 7 hrs of incubation. Moreover, the maximum decrease of H_2 receptor numbers induced by forskolin is comparable to the maximum agonist-mediated H_2 receptor downregulation.

Agonist-induced receptor downregulation is a commonly occurring regulatory process of the large family of GPCRs (see for references reviews Collins, 1993, Lohse, 1993). Enhanced degradation and/or decreased synthesis of the receptor protein are thought to contribute to receptor downregulation (Collins, 1993, Lohse, 1993). Agonist-induced downregulation of GPCRs is often accompanied by a decline of receptor mRNA levels, presumably contributing to the overall reduction in receptor number and responsiveness (Hadcock and Malbon, 1993). Indeed, incubation of CHOrH_2 cells with histamine or forskolin resulted in a transient decrease of H_2 receptor mRNA levels (70% reduction) within 4 hrs, which was followed by a gradual increase of H_2 receptor mRNA to 50% of control mRNA levels in the following hours. The initial 70% reduction of H_2 receptor mRNA is sufficient to explain the 50% reduction of the H_2 receptor numbers, since a relatively short 4 hrs treatment of CHOrH_2 cells with histamine followed by 20 hrs of wash-out already led to 50% H_2 receptor downregulation. The reduced H_2 receptor mRNA levels, 50% of the control levels, at later time points represent a new steady-state level of receptor mRNA to maintain the downregulated state of H_2 receptors. The reduction of H_2 receptor mRNA is most likely explained by post-transcriptional events, such as receptor mRNA destabilization. For e.g. the β_2AR and thrombin receptor in DDT_1MF-2 smooth muscle cells, the endothelin ET_B receptor in ROS17/2 rat osteosarcoma cells, and also for the β_2AR and muscarine m1 receptor expressed into CHW and CHO cells respectively, the decline in receptor mRNA has been ascribed to destabilization of the mRNA (Bouvier *et al.*, 1989, Hadcock and Malbon, 1989, Lee *et al.*, 1994, Sakurai *et al.*, 1992, Tholanikunnel *et al.*, 1995). In the presence of actinomycin D, breakdown of the H_2 receptor mRNA in CHOrH_2 cells was significantly stimulated upon histamine-treated and forskolin-treatment compared to non-treated cells. Recently, it was shown that a so-called M_r 35,000 β-adrenergic receptor mRNA-binding protein (β-ARB), involved in the destabilization of β_2-adrenergic receptor mRNA, also recognizes other GPCR transcripts (Tholanikunnel *et al.*, 1995). As such, our observations of H_2 receptor mRNA destabilization fits well in an apparently general mechanism of β-ARB-mediated regulation of GPCR mRNA (Port *et al.*, 1992, Tholanikunnel *et al.*, 1995).

For the β_2AR, the most extensively studied GPCR, receptor downregulation is ascribed to two pathways: an agonist-dependent, PKA-independent, and a PKA-dependent process (Collins, 1993, Lohse, 1993). Evidence for a PKA-independent pathway was obtained by studies which showed unaffected profiles of β_2AR downregulation in mutant S49 mouse

lymphoma cells defective in signal transduction components (Allen *et al.*, 1989, Mahan *et al.*, 1985, Shear *et al.*, 1976, Su *et al.*, 1980). Receptor-G_s-coupling seems to be important for the process of β_2AR downregulation, since defects in this coupling introduced by mutations of the receptor or G_s-protein have lead to impaired β_2AR downregulation (Campbell *et al.*, 1991, Hadcock and Malbon, 1988, Mahan *et al.*, 1985, Shear *et al.*, 1976, Su *et al.*, 1980). Agents responsible for the elevation of intracellular levels cAMP, such as forskolin and IBMX, or cAMP analogues, e.g. dibutyrylcAMP, were shown to induce β_2AR downregulation as well, providing evidence for the existence of cAMP-dependent receptor downregulation (Bouvier *et al.*, 1989, Collins, 1993, Hadcock and Malbon, 1988). In CHW cells the time course of the cAMP-promoted downregulation of the β_2AR was much slower than the β-agonists-induced downregulation, suggesting that distinct pathways can lead to downregulation of the β_2AR (Bouvier *et al.*, 1989). Yet, PKA-dependent phosphorylation of the β_2AR appears to enhance downregulation, since receptor mutants lacking PKA phosphorylation sites showed impaired agonist-induced downregulation (Bouvier *et al.*, 1989). Taken together, β_2AR receptor downregulation seems to require receptor-G_s coupling for the initial loss of receptor binding sites, while the cAMP-dependent decrease of receptor mRNA levels serves to maintain the downregulated state by establishing a new steady-state of receptor expression (Collins, 1993). The underlying biochemical mechanisms responsible for each of these events is, however, unclear so far.

In our study on CHOrH_2 cells, comparable time courses and a maximum extent of histamine-induced and forskolin-induced H_2 receptor downregulation as well as H_2 mRNA downregulation suggest the involvement of cAMP in the process of agonist-induced H_2 receptor downregulation. In order to assess the role of cAMP in the process of agonist-induced H_2 receptor downregulation, we constructed a mutant H_2 receptor which showed impaired G-protein coupling. Recently, Moro *et al.* (1993) have shown that hydrophobic amino acids within a highly conserved GPCR motif DRYXXV(I)XXPL (X is any amino acid and L is leucine or other lipophilic amino acid) in the second intracellular loop are involved in receptor-G-protein coupling (Moro *et al.*, 1993). In the H_2 receptor protein a DRYCAVTDPL sequence is found at an equivalent position of the highly conserved motif (Ruat *et al.*, 1991). Substitution of the Leu^{124} residue to by an alanine residue had no effect on H_2 receptor expression nor on H_2 antagonist binding properties. However, the mutation induced a marked impairment of the ability of the receptor to physically couple to its G-protein as assessed by alterations in its agonist-binding parameters (disappearance high affinity binding site, no detectable GTPγS shift). The physical uncoupling of the $H_2Leu^{124}Ala$ mutant was paralleled by a functional uncoupling, characterized by an impairment of the histamine-induced cAMP production (160-fold reduction of the EC_{50} value and 55% decrease of the maximal cAMP response). These findings are in agreement with the functional uncoupling reported by Moro *et al.* (1993) after mutation of a hydrophobic amino acid at similar position in the muscarine m1, m3 and β_2-adrenergic receptor.

Interestingly, long-term exposure of CHOr$H_2Leu^{124}Ala$ cells to 0.1 μM and 1 μM of histamine, concentrations that do *not* elicit cAMP production, resulted in a significant

reduction of [^{125}I]-APT binding sites, indicating that a cAMP-independent pathway is involved in the observed agonist-induced H_2 receptor downregulation in CHOrH_2Leu^{124}Ala cells. Previous findings in mutant S49 mouse lymphoma cells defective in signal transduction components also showed the existence of cAMP-independent pathways in the agonist-induced downregulation (Allen *et al.*, 1989, Mahan *et al.*, 1985, Shear *et al.*, 1976, Su *et al.*, 1980). However, it should be noted that the EC_{50} value of histamine-induced-H_2 receptor downregulation was shifted 16-fold to the right for the H_2Leu^{124}Ala receptor compared to the wild-type receptor. These data suggest that agonist-induced H_2 receptor downregulation depends on intact receptor-G-protein coupling. As already stated earlier, previous findings for the β_2AR have shown that defective receptor-G_s coupling leads to impaired receptor downregulation (Campbell *et al.*, 1991, Hadcock and Malbon, 1988, Mahan *et al.*, 1985, Shear *et al.*, 1976, Su *et al.*, 1980).

Moreover, no decrease in H_2Leu^{124}Ala mRNA levels was observed after 24 hrs incubation of CHOrH_2Leu^{124}Ala cells with 1 μM histamine. These data indicate that the reduction of H_2 receptor mRNA levels in CHO cells is cAMP-dependent, as generally accepted (Hadcock and Malbon, 1989). Remarkably, both the maximum histamine-induced and forskolin-induced downregulation of H_2Leu^{124}Ala receptor were found to be more pronounced than for the wild-type H_2 receptor, suggesting that the mutated receptor has become more susceptible to receptor downregulation. Although we do not have an explanation for this finding, we hypothesize that the Leu^{124}Ala mutation induces a conformational change in the second intracellular loop of the H_2 receptor protein, causing an uncoupling from the G_s protein but also an increase of the accessibility of molecular entities involved in receptor degradation. Recent studies with the parathyroid hormone receptor (Huang *et al.*, 1995) and β_1-adrenergic receptor (Green and Liggett, 1994) support this hypothesis. Small changes in the conformation of intracellular receptor domains have been shown to augment receptor internalization (Green and Liggett, 1994, Huang *et al.*, 1995). Unfortunately, no data on receptor downregulation are available for these mutant receptors (Green and Liggett, 1994, Huang *et al.*, 1995).

In conclusion, for the first time we have demonstrated that the histamine H_2 receptor is downregulated by prolonged treatment with H_2 agonists. Elevation of cAMP by long-term incubation of CHOrH_2 cells with forskolin, CTX and IBMX, is also shown to induce H_2 receptor downregulation. These data suggest the involvement of protein kinase A in the process of H_2 receptor downregulation and provides a mechanism for heterologous H_2 receptor regulation. Also H_2 receptor mRNA levels were rapidly downregulated upon both histamine-treatment and forskolin-treatment, thereby contributing to the observed decrease of H_2 receptor binding sites. Substitution of the hydrophobic amino acid leucine^{124}, located within the highly conserved G-protein coupling motif DRYXXV(I)XXPL in the second intracellular loop of the H_2 receptor, by an alanine generated a mutant receptor with impaired ability to couple to its G-protein. Interestingly, the H_2 Leu^{124}Ala mutant receptor was still downregulated by histamine, at concentrations which showed no increase of cAMP, thereby providing evidence for a cAMP-independent pathway in the process of agonist-induced H_2 receptor

downregulation. Thus, H_2 receptor downregulation appears to be induced by two distinct pathways, a cAMP-dependent and cAMP-independent pathway.

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Chapter 6

The C-terminal tail of the histamine H_2 receptor contains positive and negative signals important for signal transduction and receptor regulation

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In the present study we constructed 3 different truncated H_2 receptor mutants to examine the role of the C-terminal tail in H_2 receptor regulation. Stable transfection of H_2 receptor mutants, truncated at position 307 and 341 (H_2T307 and H_2T341), in Chinese Hamster Ovary (CHO) cells resulted in detectable [^{125}I]-iodoaminopotentidine ([^{125}I]-APT) binding, whereas no detectable [^{125}I]-APT binding of the H_2 receptor truncated at position 295 (H_2T295) was observed in CHO nor after transient transfection in HEK-293 cells. Thus the amino acids prior to position 307 appear to be necessary for proper receptor transport or folding. Ligand binding properties of the tested H_2 antagonists and histamine (formation of a ternary complex) and histamine-induced signalling were not affected by truncation of the C-terminal tail by 51 amino acids, supporting previous findings that the ligand binding domain mainly resides in the transmembrane domains of the H_2 receptor protein. Yet, removal of only 17 amino acids of the C-terminal tail generated a mutant receptor (H_2T341) which was able to form a ternary complex but was unable to fully activate the G_s-protein upon histamine exposure (70% reduction of the maximal histamine-induced cAMP response).

Desensitization, characterized by attenuated production of cAMP upon increase of the histamine incubation time, was not affected by C-terminal tail truncation of the H_2 receptor, suggesting that the serine and threonine residues in the C-terminal tail are not implicated in the mechanism underlying short-term H_2 receptor desensitization. Truncation of the C-terminal tail of the H_2 receptor by 51 amino acids, in contrast to most other GPCRs resulted in the generation of a mutant H_2 receptor (H_2T307) which was more susceptible to agonist-induced receptor downregulation, but not to the cAMP-dependent H_2 receptor downregulation. These data indicate that the cAMP-dependent and independent pathways of H_2 receptor downregulation appear to be mediated by different regulatory processes. Taken together, in this study we identified regions in the C-terminal tail of the H_2 receptor, which appear to act as positive and/or negative signals in H_2 receptor signalling and H_2 receptor regulation.

Introduction

Recent studies have shown that the histamine H_2 receptor, like other G-protein coupled receptors (GPCRs), is susceptible to receptor regulation (Arima *et al.*, 1993, Fukushima *et al.*, 1994, Smit *et al.*, 1994, Chapter 4, 5). At least three mechanistically and temporarily distinct processes contribute to the overall phenomenon of desensitization of the GPCRs (Collins, 1993, Lohse, 1993, Savarese and Fraser, 1992). The first involves a rapid (secs to mins) uncoupling of these receptors from their effector system (desensitization), the second is characterized by the translocation of these receptors away from the cellular surface within minutes (internalization) and the third is characterized as a reduction of receptor binding sites occurring after hours of agonist exposure (downregulation) (for references see reviews Collins, 1993, Lohse, 1993, Savarese and Fraser, 1992).

In view of the potential use of H_2 agonists in patients sufferering from congestive heart failure (Felix *et al.*, 1991) detailed investigation of H_2 receptor regulation has gained interest.

However, knowledge regarding H_2 receptor regulation is limited and has only recently become available. The endogenous histamine H_2 receptor in the human U937 lymphoma cell line and the human gastric carcinoma cell line MKN-45 as well as the canine H_2 receptor expressed in Chinese Hamster Ovary cells (CHO cells) were found to undergo rapid receptor desensitization upon agonist exposure (Arima *et al.*, 1993, Fukushima *et al.*, 1994, Smit *et al.*, 1994). Furthermore, long-term exposure of the rat histamine H_2 receptor expressed in CHO cells to histamine was shown to result in dose- and time-dependent H_2 receptor downregulation (Chapter 5). The process of agonist-induced H_2 receptor downregulation appeared to be mediated both by cAMP-dependent and cAMP-independent pathways, as was shown by studies with H_2 receptor mutants (Chapter 5).

The molecular entities and H_2 receptor domains involved in H_2 receptor regulation are still unknown. Yet, the involvement of various domains and specific amino acids in GPCR regulation has been studied for several other members of the family of GPCRs. In particular the third intracellular loop and the C-terminal tail of the GPCRs are important for receptor regulation (see reviews Collins, 1993, Lohse, 1993, Savarese and Fraser, 1992). These domains contain various serine and threonine residues which may serve as potential phosphorylation sites for serine/threonine kinases. Phosphorylation of serine and threonine residues in the carboxyl terminal region of many GPCRs by specific receptor kinases and in the third intracellular loop and/or carboxyl terminus by second messenger-dependent kinases is thought to direct rapid uncoupling from the signal transduction pathway (Bouvier *et al.*, 1988, Hausdorff *et al.*, 1989, Hausdorff *et al.*, 1991, Lattion *et al.*, 1994, Liggett *et al.*, 1989, Roth *et al.*, 1991, Takano *et al.*, 1994). Also for agonist-induced receptor internalization the importance of serine/threonine-rich regions in the C-terminal tail of the GPCRs has been reported, suggesting that a phosphorylation-driven mechanism participates in this process (Benya *et al.*, 1993, Hausdorff *et al.*, 1991, Hunyady *et al.*, 1994, Nussenzveig *et al.*, 1993, Thomas *et al.*, 1995). Finally, the C-terminal tail of many GPCRs appears to be an important structural determinant for the process of receptor downregulation. Truncation or site-directed mutagenesis of serine-, threonine- or tyrosine residues of the C-terminal tail of, for example, the β_2-adrenergic receptor and muscarine m3 receptor were shown to decrease the rate of receptor downregulation (Bouvier *et al.*, 1989, Campbell *et al.*, 1991, Cheung *et al.*, 1989, Valiquette *et al.*, 1990, Yang *et al.*, 1993). In addition, isoforms of the somatostatin (mSSTR2A and mSSTR2B) (Vanetti *et al.*, 1993) or of the prostaglandin E_2 receptor ($EP_{3\alpha}$ and $EP_{3\beta}$), which differ in their C-terminal tail (Negishi *et al.*, 1993), showed different patterns of receptor downregulation upon exposure to the respective agonist. The role of the C-terminal tail in receptor downregulation appears to be complex. For the avian β_1-receptor the C-terminal tail seems to prevent receptor downregulation as truncation of the receptor eliminates the insensitivity of the β_1-adrenergic receptor towards receptor downregulation (Hertel *et al.*, 1990, Parker and Ross, 1991).

In view of the above findings the C-terminal tail should be considered as an important structural domain for GPCR regulation. Especially serine/threonine containing domains appear to be involved in the various processes of GPCR regulation (Benya *et al.*, 1993, Bouvier *et al.*, 1988, Bouvier *et al.*, 1989, Hausdorff *et al.*, 1989, Hausdorff *et al.*, 1991, Hunyady *et al.*, 1994, Liggett *et al.*, 1989, Nussenzveig *et al.*, 1993, Roth *et al.*, 1991, Thomas *et al.*, 1995, Yang *et al.*, 1993). Since the H_2 receptor contains 11 serine and threonine residues in the C-terminal tail, we found it tempting to speculate that this structural element is involved in the observed H_2 receptor regulation. We therefore constructed three receptor mutants truncated at different positions of the C-terminus and we stably expressed them in CHO cells (Fig. 1). The H_2 receptor mutant truncated at position 295 lacks all serine/threonine residues localized in the C-terminus of the receptor, whereas the H_2 receptor mutant truncated at positions 307 and 341 lack 10 and 3 of these residues respectively. Using these receptor mutants we first examined whether truncation affected ligand binding and the H_2 receptor-mediated signal transduction. Various reports have shown that C-terminal truncation resulted in decreased G-protein coupling (Josiah *et al.*, 1994, Kosugi and Mori, 1994, Ohyama *et al.*, 1992, Sasakawa *et al.*, 1994), whereas others showed no effect on ligand binding nor signal transduction (Benya *et al.*, 1993, Bouvier *et al.*, 1988, Koch *et al.*, 1994, Nussenzveig *et al.*, 1993, Thomas *et al.*, 1995, Zhu *et al.*, 1993). Finally, we examined whether the C-terminus of the H_2 receptor is a structural domain crucial for agonist-, cAMP-independent as well as cAMP-induced receptor regulation.

Materials and methods

Cell culture CHO cells expressing the rat histamine H_2 receptor ($CHOrH_2$) (Traiffort *et al.*, 1992) and the truncated H_2 receptors ($CHOrH_2T341$ and $CHOrH_2T307$) were grown at 37°C in a humified atmosphere with 5% CO_2 in Dulbeco's Modified Eagle's Medium (DMEM), containing 10% (vol/vol) dialyzed foetal calf serum and supplemented with 2 mM L-glutamine, MEM amino acids, 50 IU/ml penicillin and 50 µg/ml streptomycin. *Site-directed mutagenesis* cDNA's encoding truncated H_2 receptor proteins were constructed by means of the Polymerase Chain Reaction (PCR), using the gene of the rat H_2 receptor as a template (Ruat *et al.*, 1991). Different 3' primers were designed to introduce a stop-codon at the desired position (amino acid position 295, 307 and 341) followed by a *Bgl*II site. The oligonucleotide S1 (5'-GGGAAGCTTGGCCCCAGAATGGA-GCCCAATGGCACAGT), corresponding to nucleotides -9 to 21 (Ruat *et al.*, 1991) and a *Hind*III site (underlined) and oligonucleotides, AT341 (5'-CGGAGATCTTTAGAGCTTCAAGGGCTTCTCCTC), corresponding to nucleotides 1003-1026 of the complementary strand, AT307 (5'-CGGAGATCTTTATGCGA-ACTTGCAGTGGAAGAG), corresponding to nucleotides 901-921 of the complementary strand and AT295 (5'-CGGAGATCTTTAGCGGAAGTCTCTGTTGAGAGC), corresponding to nucleotides 865-885 of the complementary strand, all containing a *Bgl*II site (underlined) with one or two nucleotide changes to introduce a stop codon were synthesized on an Applied Biosystems DNA synthesizer (model 381A). Using 100 ng $pSVrH_2$ (Traiffort *et al.*, 1992) as a template, 0.4 µM S1, 0.4 µM AT341, 200 µM dNTP's and 2.5 U Amplitaq (Perkin Elmer) a 1035 bases DNA fragment of the H_2T341 receptor mutant was amplified in 100 µl using 20 cycles at 95°C for 1 min, 56°C for 1 min and 72°C for 1 min and a final extension at 72°C for 10 mins. Similar conditions were used to amplify respectively a 930 and 894 bases fragment for the construction of the H_2T307 and H_2T295 receptor mutants. The obtained PCR products were gel-purified and restricted with *Hind*III/*Bgl*II (Boehringer) and cloned into the plasmid pSP73 (Promega) in order to verify the complete nucleotide sequence using the dideoxy-chain termination method with the Sequenase kit (USB). Subsequently, the coding sequence of H_2T341, H_2T307 and H_2T295 receptor was subcloned into the eukaryotic pSV expression vector. CHO cells, deficient in dihydrofolate reductase, were stably transfected with 15 µg expression vectors $pSVrH_2T341$,

pSVrH_2T307 and pSVrH_2T295 using Transfectam (Promega).

Biochemical measurements The determination of H_2 receptor binding and levels of cAMP were performed as described in Chapter 5.

RNA slot blot analysis RNA was analyzed as described in Chapter 5.

Statistical analysis All data shown are expressed as mean ± standard error (mean ± s.e. mean) of at least three independent experiments. Statistical analysis was carried out by Student's *t*-test. P-values < 0.05 were considered to indicate a significant difference.

Chemicals Histamine dihydrochloride, isobutylmethylxanthine (IBMX), cyclic AMP (cAMP), forskolin, were obtained from Sigma Chemical Company (USA). [2,8-^{3}H]-cAMP (40 Ci/mmol) was obtained from Amersham. Aminopotentidine was taken from laboratory stock. Gifts of cimetidine (SmithKline Beecham, United Kingdom) and ranitidine dihydrochloride (Glaxo, United Kingdom) are gratefully acknowledged.

Results

Pharmacological characterization of the truncated H_2 receptor mutants

A series of cDNAs, encoding rat histamine H_2 receptor proteins truncated at the C-terminus (Fig. 1), were constructed by PCR and stably expressed in CHO cells as described in the section "Methods". Individual clones were isolated and assayed for [^{125}I]-APT binding. With the exception of the shortest construct (H_2T295) the other receptor mutants were functionally expressed. Transient expression of the H_2T295 mutant, inserted into a pRK$_5$ vector, in human embryonal kidney HEK-293 cells did not show detectable [^{125}I]-APT binding either (data not shown). H_2 mRNA studies, on the other hand, revealed synthesis of H_2T295 mRNA in transiently transfected HEK-293 cells (data not shown). For the other mutants clonal cell lines expressing comparable amounts of [^{125}I]-APT binding as CHOrH_2 cells, expressing the wild-type rat H_2 receptor, were chosen for further analysis and referred to as CHOrH_2T341 and CHOrH_2T307.

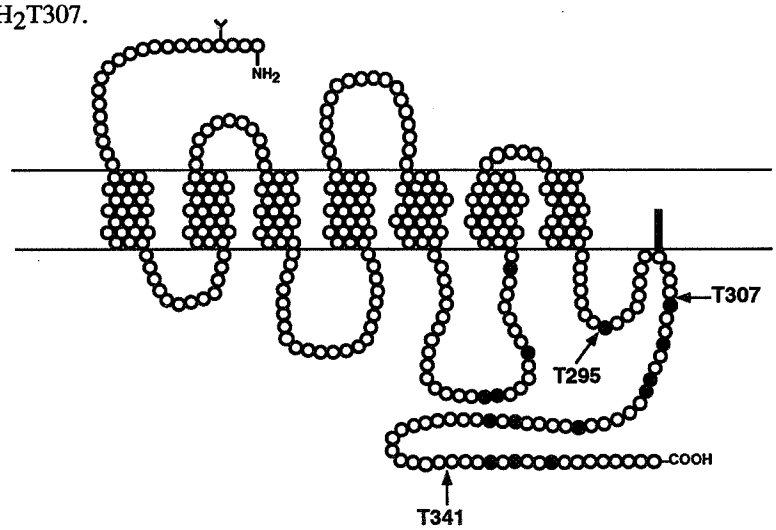

Fig. 1. Schematic representation of the rat histamine H_2 receptor. The histamine H_2 receptor was truncated at positions 341 (H_2T341), 307 (H_2T307) and 295 (H_2T295) by insertion of a stop codon by site-directed mutagenesis as described under "Methods". Solid circles indicate the presence of either a serine or a threonine. The assumed palmitoylation and membrane anchorage of Cys^{304} is indicated.

The dissociation constant of [^{125}I]-APT for the truncated receptors (H_2T341 and H_2T307) was comparable to that of the wild-type receptor (Table 1). Moreover, the K_i values of aminopotentidine, cimetidine and ranitidine for the truncated receptors were not significantly different compared to the wild-type H_2 receptors (Fig. 2A, Table 2). The histamine displacement curves in CHOrH_2, CHOrH_2T341 and CHOrH_2T307 cells were shallow and could all be best analyzed by a two site model (Table 2, Fig. 2B, C), indicating the presence of high and low affinity binding sites. Note, however, that the percentage of high affinity binding sites for the H_2T341 mutant is significantly increased compared to the wild-type receptor (Fig. 2B, Table 2).

Table 1 Characteristics of [^{125}I]-APT binding to CHOrH_2, CHOrH_2T341 and CHOrH_2T307 cell membranes

CHOrH_2	K_d (nM)	B_{max} (fmol/mg protein)
WT	0.71 ± 0.08	1172 ± 286
T341	0.60 ± 0.08	616 ± 195
T307	0.56 ± 0.12	1041 ± 152
T295	-	not detectable

The dissociation constant (K_d) and maximum number of binding sites (B_{max}) was determined by using non-linear fitting according to a one-site binding mode. The data shown represent the mean ± s.e. mean of four independent experiments.

Table 2 Pharmacological characterization of [^{125}I]-APT binding to CHOrH_2, CHOrH_2T341 and CHOrH_2T307 cell membranes

		K_i-value		
		CHOrH_2	CHOrH_2T341	CHOrH_2T307
aminopotentidine		5.8 ± 0.9 nM	6.4 ± 1.1 nM	6.6 ± 1.0 nM
cimetidine		804 ± 53 nM	935 ± 195 nM	837 ± 17 nM
ranitidine		232 ± 45 nM	194 ± 39 nM	244 ± 54 nM
histamine	$K_{i,\ high}$	3.5 ± 1.0 µM (39 ± 7%)	4.8 ± 1.5 µM (60 ± 5%) *	7.7 ± 3.2 µM (36 ± 12%)
	$K_{i,\ low}$	0.3 ± 0.04 mM (61 ± 7%)	0.6 ± 0.2 mM (40 ± 5%) *	0.4 ± 0.08 mM (64 ± 12%)

Membranes of CHOrH_2, CHOrH_2T341 and CHOrH_2T307 cells were incubated with 0.3 nM [^{125}I]-APT in the presence of the indicated drugs at increasing concentrations. K_i values were obtained from the respective IC_{50} values. Data shown are mean ± s.e.mean from at least three independent experiments. Values in parentheses indicate the relative densities of the respective sites. The asterisk indicates a significant difference ($p < 0.05$) compared to CHOrH_2 cells.

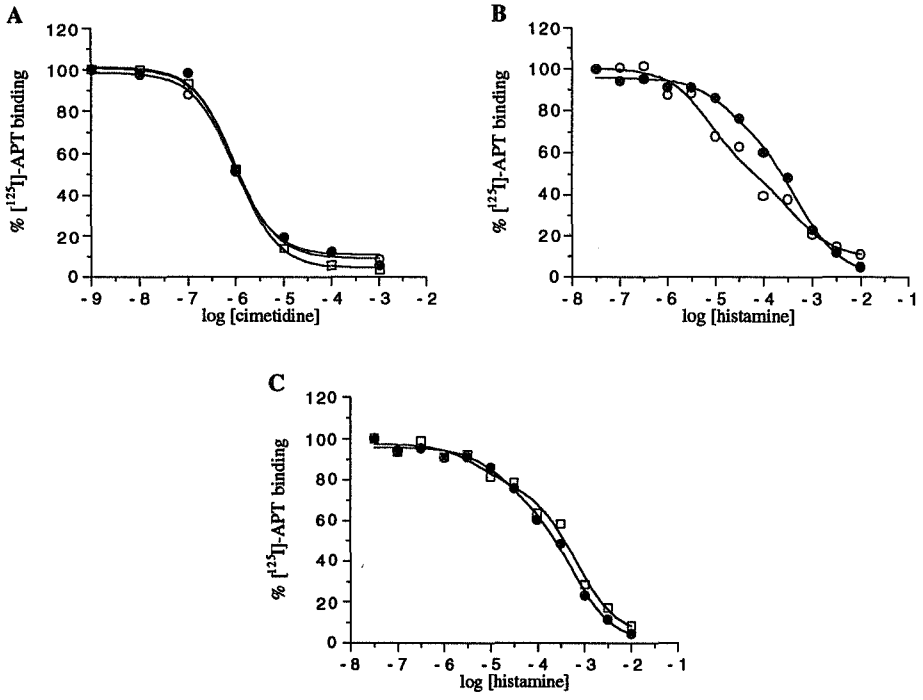

Fig. 2. Pharmacological profile of [^{125}I]-APT binding on CHOrH$_2$, CHOrH$_2$T341 and CHOrH$_2$T307 cell membranes. Displacement of 0.3 nM [^{125}I]-APT binding by increasing concentrations of cimetidine (A) and histamine (B, C) in CHOrH$_2$ (filled circles), CHOrH$_2$T341 (open circles) and CHOrH$_2$T307 (open squares) cell membranes. Data shown are mean values of triplicate determinations from a typical experiment. Similar experiments were obtained in three other independent experiments.

cAMP accumulation of cAMP in CHOrH$_2$, CHOrH$_2$T341 and CHOrH$_2$T307 cells

In order to confirm that the truncated receptors stimulate the signal transduction pathway, we determined the ability of histamine to induce a rise in cAMP in these cell lines. In all cell lines histamine elicited an increase in cAMP production (Table 3). Yet, the maximal increase of cAMP in CHOrH$_2$T341 cells was markedly impaired (~ 70% reduction). The observed reduction in cAMP production in CHOrH$_2$T341 cells cannot be ascribed to a decrease in adenylyl cyclase activity as 10 μM forskolin induced a cAMP response comparable to that in CHOrH$_2$ cells (Table 3). The EC_{50} values for the histamine-induced cAMP response in CHOrH$_2$T341 and CHOrH$_2$T307 cells, were the same, however, as measured in CHOrH$_2$ cells (Table 3).

Table 3 Histamine- and forskolin-induced cAMP production in $CHOrH_2$, $CHOrH_2T341$ and $CHOrH_2T307$ cells

	$CHOrH_2$	$CHOrH_2T341$	$CHOrH_2T307$
histamine			
basal (pmol/well)	3 ± 1	2 ± 0.4	1 ± 0.5 *
EC_{50} (nM)	36 ± 2	42 ± 12	56 ± 14
E_{max} (pmol/well)	48 ± 8	15 ± 3 *	53 ± 5
10 μM forskolin (pmol/well)	59 ± 10	54 ± 4	34 ± 1 *

Cells were incubated with increasing concentrations of histamine or 10 μM forskolin for 10 mins at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes, pH 7.4. Data represent the mean ± s.e.mean of six to seven independent experiments. The asterisk indicates a significant difference ($p < 0.05$) from $CHOrH_2$ cells.

When $CHOrH_2$ cells were exposed to 100 μM histamine, cAMP accumulated within 10 mins, which was followed by an attenuation of the cAMP response at later time points (Fig. 3A). The lack of increase in cAMP production at later time points cannot be ascribed to impaired adenylyl cyclase activity, as 10 μM forskolin continued to elevate the production of cAMP to levels higher than observed for histamine (Fig. 3A). The decline in 100 μM histamine-induced cAMP accumulation in time was also observed in $CHOrH_2T341$ and $CHOrH_2T307$ cells.

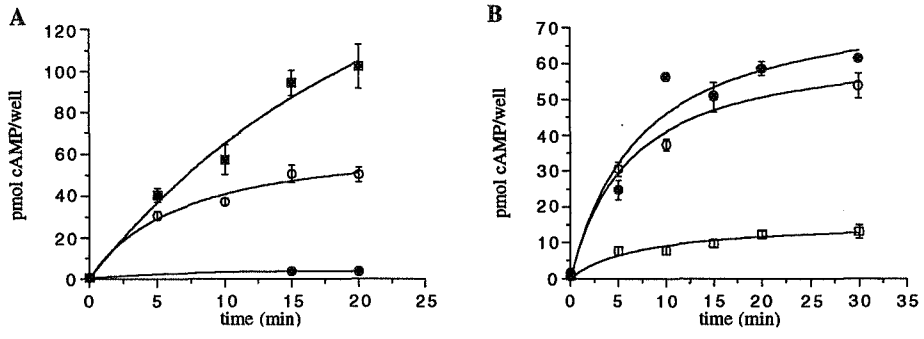

Fig. 3. Time course of the histamine-induced cAMP accumulation in $CHOrH_2$, $CHOrH_2T341$ and $CHOrH_2T307$ cells. A, $CHOrH_2$ cells were incubated with DMEM (filled circles), 100 μM histamine (open circles) or 10 μM forskolin (filled squares) for increasing periods of time at 37°C in the presence of 300 μM IBMX. B, $CHOrH_2$, (open circles) $CHOrH_2T341$ (open squares) and $CHOrH_2T307$ (filled circles) cells were incubated with 100 μM histamine for increasing periods of time at 37°C in the presence of 300 μM IBMX. Data shown are mean values ± s.e. mean of three to four independent experiments determined in triplicate.

Histamine-induced downregulation of the wild-type and truncated H_2 receptors

As was shown in previous studies (Chapter 5) exposure of $CHOrH_2$ cells to 100 μM of histamine for prolonged periods of time resulted in a time-dependent decrease of [^{125}I]-APT binding sites of 46 ± 8% (mean ± s.e.mean, n = 3) (Fig. 4). Similar results were obtained when incubating $CHOrH_2T341$ cells for increasing periods of time with 100 μM histamine (Fig. 4) (reduction 62 ± 5%, mean ± s.e.mean, n = 3). Remarkably, a significantly more pronounced reduction in [^{125}I]-APT binding sites was observed (80 ± 3%, mean ± s.e.mean, n = 3, $p < 0.05$) when $CHOrH_2T307$ cells were exposed to 100 μM histamine for increasing periods of time, although the time course of receptor downregulation remained the same (Fig. 4). Also the potency of histamine to induce H_2 receptor downregulation was not affected by the truncation. Long-term exposure of $CHOrH_2$ cells, $CHOrH_2T341$ and $CHOrH_2T307$ cells to increasing concentrations of histamine resulted in a dose-dependent decrease of [^{125}I]-APT binding sites, with comparable EC_{50} values (Table 4).

The H_2 receptor needs not to be activated by an agonist in order to be downregulated (Inset Fig. 4) (Chapter 5). Also in $CHOrH_2T341$ and $CHOrH_2T307$ cells 10 μM forskolin induced downregulation of the H_2 receptor (Inset Fig. 4). The extent of H_2 receptor downregulation was similar in the three cell lines.

Downregulation of H_2 receptor mRNA levels in $CHOrH_2$ and $CHOrH_2T307$ cells

Earlier studies have demonstrated that agonist-induced H_2 receptor downregulation in $CHOrH_2$ cells is accompanied by a decrease in H_2 receptor mRNA levels (Chapter 5). In order to investigate whether the increased downregulation observed in $CHOrH_2T307$ cells is a result from increased attenuation of H_2 receptor mRNA levels, H_2T307 receptor mRNA levels were determined at different time intervals. Incubation of $CHOrH_2$ and $CHOrH_2T307$ cells with 100 μM histamine resulted for both cell lines in a transient decrease of H_2 receptor mRNA levels within 4 hrs, followed by a gradual increase of H_2 receptor mRNA levels to 50% of control in the following hours (Fig. 5). No change in H_2 receptor mRNA downregulation could be observed in the $CHOrH_2T307$ cells when compared to the $CHOrH_2$ cells.

Table 4 Histamine-induced decrease of [^{125}I]-APT binding in $CHOrH_2$, $CHOrH_2T341$ and $CHOrH_2T307$ cells

	$CHOrH_2$	$CHOrH_2T341$	$CHOrH_2T307$
EC_{50} (nM)	18 ± 6	20 ± 6	28 ± 2
E_{max} (%)	46 ± 8	62 ± 5	80 ± 3*

$CHOrH_2$ cells, $CHOrH_2T341$ and $CHOrH_2T307$ cells were exposed to increasing concentrations of histamine for 24 hrs and the [^{125}I]-APT binding in membranes was measured. The maximum extent of decrease of [^{125}I]-APT binding is expressed as a percentage of [^{125}I]-APT binding measured in non-treated cells (E_{max} %). The data represent the mean ± s.e.mean of three to seven independent experiments.

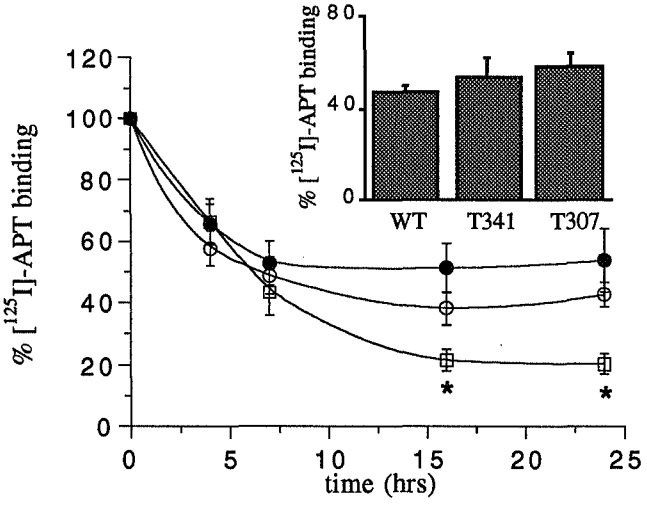

Fig. 4. Time-dependent decrease of [^{125}I]-APT binding in CHOrH_2, CHOrH_2T341 and CHOrH_2T307 cells by histamine. CHOrH_2 (filled circles), CHOrH_2T341 (open circles) and CHOrH_2T307 (open squares) cells were incubated with 100 μM histamine for the indicated time periods and [^{125}I]-APT binding in membranes was measured. The [^{125}I]-APT binding is expressed as a percentage of [^{125}I]-APT binding measured in non-treated cells (*: $p < 0.05$). The data shown represent the mean ± s.e.mean of three independent experiments Inset, Forskolin-induced decrease of [^{125}I]-APT binding sites in CHOrH_2, CHOrH_2T341 and CHOrH_2T307 cells. Cells were incubated with 10 μM forskolin for 24 hrs and [^{125}I]-APT binding in the membranes was measured. The data shown represent the mean ± s.e. mean of 4 independent experiments.

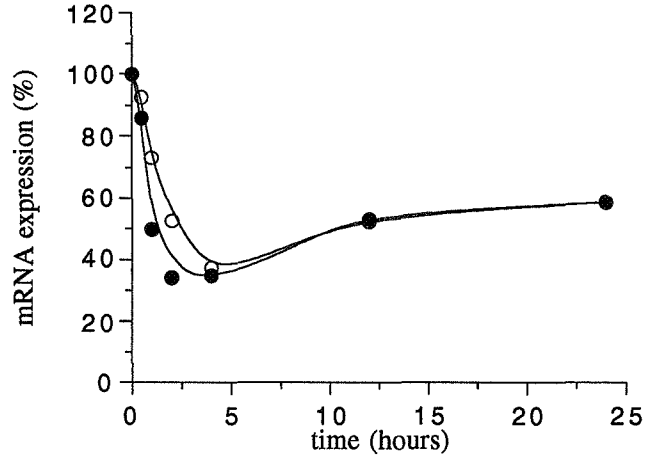

Fig. 5. Histamine-induced modulation of H_2 receptor mRNA levels in CHOrH_2 and CHOrH_2T307 cells. CHOrH_2 (filled circles) and CHOrH_2T307 (open circles) cells were incubated for the indicated times with 100 μM histamine. Cells were harvested, total RNA was extracted and H_2 receptor mRNA expression was quantified by means of a RNA slot blot assay as described in Chapter 5. Data shown are the mean values of duplicate determinations from a typical experiment. Similar results were obtained in another independent experiment.

Discussion

Recent reports have shown that the H_2 receptor is susceptible to short- and long-term receptor regulation (Arima *et al.*, 1993, Fukushima *et al.*, 1994, Smit *et al.*, 1994) (Chapter 5). As C-terminal domains are considered to be important for GPCR regulation (see reviews Collins, 1993, Lohse, 1993, Savarese and Fraser, 1992), we constructed 3 different truncated H_2 receptor mutants to examine the role of the C-terminal tail in H_2 receptor regulation. Stable transfection of H_2 receptor mutants, truncated at positions 307 and 341 (H_2T307 and H_2T341), in CHO cells resulted in detectable [^{125}I]-APT binding, whereas no detectable [^{125}I]-APT binding of the H_2 receptor truncated at position 295 (H_2T295) was observed in CHO nor after transient transfection in HEK-293 cells. Although no [^{125}I]-APT binding of the H_2T295 mutant was detected, synthesis of H_2T295 mRNA in HEK-293 cells was observed, suggesting that the H_2T295 receptor protein is not efficiently transported and/or inserted into the membrane. Similar observations or lowered receptor expression were reported after C-terminal tail truncation of various other GPCRs, indicating that the region adjacent to transmembrane (TM7) may serve as a general domain critical for GPCR expression in the membrane (Alblas *et al.*, 1995, Cheung *et al.*, 1989, Josiah *et al.*, 1994, Klos *et al.*, 1994, Moro *et al.*, 1993, Schneider *et al.*, 1994, Thomas *et al.*, 1995). In most GPCRs a highly conserved cysteine residue is found in this domain (position 304 in the H_2 receptor) (Savarese and Fraser, 1992). For several GPCR members this residue has been shown to be palmitoylated (Kennedy and Limbird, 1994, Ng *et al.*, 1993, Ng *et al.*, 1994, Ovchinnikov *et al.*, 1988) and this structural modification is thought to result in membrane anchorage of the C-terminal tail and thereby in the formation of an additional intracellular loop (O'Dowd *et al.*, 1989). Based on the observed loss of receptor expression after truncation of the C-terminal tail prior to the conserved cysteine, one might speculate that the elimination of this membrane anchorage is responsible for an incorrect positioning of TM7 in the membrane or for an incorrect initial insertion of the receptor into the membrane. However, there are also studies describing that C-terminal tail truncation prior to the cysteine residue (Nussenzveig *et al.*, 1993, Takano *et al.*, 1994) did not affect receptor expression. Moreover, some GPCRs, like the α_2C4 receptor, do not even possess a cysteine in their C-terminal tail (Eason *et al.*, 1994). The reason for this apparent discrepancy between different members of the GPCR family is not clear at this moment, but suggests that perhaps other structural features adjacent to TM7 (residues 295-307) are of importance for proper receptor expression.

The C-terminal region of the H_2 receptor from amino acid position 307 does not seem to be important for the binding of H_2 receptor ligands to the receptor as the binding properties of various H_2 antagonists and histamine were unaffected. These results confirm previous findings that the binding of histamine to the histamine receptors mainly occurs in the transmembrane domains (Gantz *et al.*, 1992, Leurs *et al.*, 1994, Ohta *et al.*, 1994). The C-terminal tail of some GPCRs appears to be involved in G-protein coupling, as G-protein coupling was found

to be reduced upon truncation of the C-terminal tail (Josiah *et al.*, 1994, Kosugi and Mori, 1994, Ohyama *et al.*, 1992, Sasakawa *et al.*, 1994). For the H_2 receptor, no difference in the ability of histamine to stimulate the H_2T307 was observed, suggesting that the major part of the C-terminal region (51 amino acids) is not involved in the activation of the signal transduction pathway. Surprisingly, however, truncation of the C-terminal tail with only 17 amino acids (H_2T341) attenuated the capacity of histamine to induce production of cAMP (~ 70% reduction), even though high affinity binding for histamine was observed. Even a small increase in the percentage of high affinity binding for the H_2T341 receptor was observed. Yet, the EC_{50} value of the histamine-induced cAMP response in $CHOrH_2T341$ cells (42 nM) did not significantly differ from the EC_{50} value recorded in $CHOrH_2$ cells (36 nM). The observed decrease in the maximum cAMP production cannot be attributed to a lower receptor expression (616 fmol/mg protein vs 1172 fmol/ mg protein for $CHOrH_2$ cells), as CHO cells expressing the wild-type receptor at a density of ~500 fmol/mg of protein showed a similar maximum histamine-induced response as that in $CHOrH_2$ cells (M.J. Smit, unpublished observations). Detection of high affinity binding sites for GPCR agonists is considered to reflect the formation of a ternary agonist-receptor-G-protein complex (De Lean *et al.*, 1980). Formation of such a ternary complex is usually thought to lead to a dissociation of the G-protein subunits which results in an effective activation of the signal transduction cascade. Our findings, however, indicate that the formation of a high affinity agonist-receptor-G-protein complex is not sufficient enough to fully activate the G_s-protein. Instead, an additional stimulatory signal appears to be required. The existence of distinct molecular determinants involved in physical coupling to and functional activation of the G_s-protein has also been reported for the β_2-adrenergic receptor upon deletion of the C-terminal region of the third intracellular loop (Hausdorff *et al.*, 1990). Also some rhodopsin receptor mutants were found to bind transducin, but they were found to be defective in catalysing the GDP dissociation from transducin (Ernst *et al.*, 1995). Since histamine was capable to fully activate the H_2T307 receptor, the region encompassing amino acids 307 to 341 seems to contain a signal motif that impairs proper G_s-protein activation. The last 17 amino acids of the H_2 receptor appear to prevent this impairment by the 307-341 region, thereby serving as a positive signal for G_s-mediated signal transduction. These findings indicate that this region of the C-terminal tail of the H_2 receptor is important for G_s-protein activation. The presence of such positive and negative signals in the C-terminal tail has also been reported for the parathyroid hormone receptor (Huang *et al.*, 1995). Different domains were found to act as stimulators, while other domains as inhibitors of the process of internalization (Huang *et al.*, 1995). Future site specific mutagenesis studies should further define which amino acids in the region encompassing 307 and 341 of the H_2 receptor are responsible for the impaired G_s-protein activation.

Subsequently, we were interested to find out whether C-terminal truncation affected H_2 receptor regulation. Previous studies have shown that both the human H_2 receptor in U937 or

MKN-45 cells and the canine H_2 receptor expressed in CHO cells are rapidly desensitized upon short-term histamine exposure (Arima *et al.*, 1993, Fukushima *et al.*, 1994, Smit *et al.*, 1994). Receptor desensitization of GPCRs often leads to blunting of agonist-induced cAMP production or inositol phosphate production in time as shown, for example, for the β_2-adrenergic receptor and the neurokinin-2 receptor (Alblas *et al.*, 1995, Liggett *et al.*, 1989). Elimination of potential phosphorylation sites in the β_2-adrenergic receptor and truncation of the neurokinin-2 receptor have been reported to result in a sustained stimulation of the respective effector enzymes compared to the transient activation seen in cells expressing wild-type receptors (Alblas *et al.*, 1995, Liggett *et al.*, 1989). We also found that the wild-type H_2 receptor response is desensitized in time upon agonist exposure. In our studies, a similar pattern of desensitization was observed in time in CHOrH_2T341 and CHOrH_2T307 cells, implying that phosphorylation of serine/threonine residues by kinases in the C-terminal tail of the H_2 receptor is not responsible for the observed desensitization. Previously, truncation of the C-terminus of the luteinizing hormone (Zhu *et al.*, 1993) and angiotensin II AT_{1A} (Thomas *et al.*, 1995), both containing various serine- and threonine residues, did not affect the pattern of agonist-induced desensitization either, indicating that the C-terminal tail of these receptors has no apparent role in desensitization. As such, different receptor domains appear to be involved in agonist-induced GPCR desensitization. The third intracellular loop of the H_2 receptor contains four more potential phosphorylation sites, which might be implicated in the observed agonist-induced desensitization. Substitution of these phosphorylation sites and/or direct phosphorylation studies with co-expression of specific receptor kinases should give more insight in the phenomenon of agonist-induced H_2 receptor desensitization.

Former studies have also shown that the H_2 receptor is susceptible to long-term receptor activation, resulting in H_2 receptor downregulation (Chapter 5). Two mechanisms seem to be involved in the process of H_2 receptor downregulation, a cAMP-dependent as well as agonist-dependent, cAMP-independent (Chapter 5). As already stated in the Introduction, the C-terminal tail of many GPCRs appears to be a structural determinant for receptor downregulation (see reviews Collins, 1993, Lohse, 1993, Savarese and Fraser, 1992). In CHOrH_2T341 cells histamine induced a time- and dose-dependent decrease of [^{125}I]-APT binding sites comparable to that in CHOrH_2 cells, even though the histamine-induced cAMP production was found to be attenuated in the mutant receptor. These data further support our previous findings (Chapter 5) describing a cAMP-independent pathway inducing H_2 receptor downregulation. The last 17 amino acids do not seem to be implicated in the process of histamine-induced H_2 receptor downregulation, although they function as a positive signal in the process of signal transduction.

The cAMP-dependent downregulation of the wild-type H_2 receptor, induced by forskolin, did not alter upon truncation of either 17 or 59 amino acids, which indicates that the C-terminal tail of the H_2 receptor is not involved in the process of the cAMP-dependent H_2 receptor downregulation. It is remarkable that in CHOrH_2T307 cells the observed agonist-induced H_2

receptor downregulation was more pronounced than in CHOrH_2 cells (80% vs 44%). Downregulation of the wild-type H_2 receptor can be partly ascribed to an increased H_2 mRNA breakdown (Chapter 5). However, the increased downregulation of the H_2T307 receptor cannot be explained by an increased H_2T307 mRNA breakdown, since no change in the pattern of H_2T307 mRNA downregulation was observed compared to the wild-type H_2 receptor. These findings are confirmed by the lack of a more pronounced effect induced by long-term forskolin treatment, assuming that H_2 mRNA downregulation process is cAMP-mediated, as generally accepted (Hadcock and Malbon, 1993, Chapter 5). The increased downregulation in CHOH_2T307 cells was rather unexpected and in contrast with the reports of, for example, the β_2-adrenergic receptor and muscarine m3 receptor, here removal of serine, threonine or tyrosines from the C-terminal tail resulted in a decrease of receptor downregulation (Bouvier *et al.*, 1989, Campbell *et al.*, 1991, Chueng *et al.*, 1989, Valiquette, *et al.*, 1990, Yang *et al.*, 1993). The only studies showing increased downregulation upon C-terminal truncation are those of the avian β_1-adrenergic receptor (Hertel *et al.*, 1990, Parker and Ross, 1991). This avian receptor has a C-terminal tail which is 59 amino acids longer than the mammalian β_1-adrenergic receptor. The authors suggested that the additional 59 amino acids act as an anchorage of the receptor to the cytoskeleton, thereby blocking signal transduction efficacy and regulatory mechanisms such as internalization and downregulation (Parker *et al.*, 1995, Wang and Ross, 1995). In view of these findings we hypothesize that the region which contains the amino acids 307 to 341 and which is found to act as a negative signal in the histamine-induced signalling, also impairs receptor downregulation. Truncation of the H_2 receptor with 51 amino acids is considered to increase the accessibility of molecular entities involved in the process of agonist-induced H_2 receptor downregulation.

In conclusion, this study has provided information whether the C-terminal tail of the H_2 receptor is involved in ligand binding, signal transduction and receptor regulation. The amino acids prior to position 307 appear to be necessary for a proper receptor transport or folding. Ligand binding properties of the tested H_2 antagonists and histamine were not affected by truncation, indicating that their binding domain mainly resides in the transmembrane domains of the H_2 receptor protein. Truncation of the major part of the C-terminal tail (51 amino acids) did not affect the histamine-induced production of cAMP, whereas removal of only 17 amino acids resulted in the formation of a receptor capable of coupling to G proteins, but unable to fully activate the G_s-protein upon histamine exposure. These data suggest that this region of the C-terminal tail of the H_2 receptor is important for G-protein activation. Truncation of the C-terminal tail did not influence the agonist-induced desensitization, indicating that the serine and threonines residues are not involved in the mechanism underlying short-term H_2 receptor desensitization. The C-terminal tail does not appear to be important in the cAMP-dependent pathway of H_2 receptor downregulation, whereas truncation of the last 51 amino acids enhanced the observed agonist-, cAMP-independent H_2 receptor downregulation. These data

suggest that the cAMP-dependent and -independent pathways of the H_2 receptor downregulation are mediated by different regulatory processes. Taken together, our findings show that the C-terminal tail of the H_2 receptor contains regions which act as positive or negative signals important for signal transduction and receptor regulation. In order to determine the exact role of various amino acids and/or receptor domains in the C-terminal tail of the H_2 receptor in these different processes, investigations including site directed mutagenesis are required.

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Chapter 7

Pharmacological characterization of the human histamine H_2 receptor stably expressed in Chinese Hamster Ovary cells

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In the present study the gene encoding the human histamine H_2 receptor was stably expressed in Chinese Hamster Ovary (CHO) cells and characterized by [^{125}I]-iodoaminopotentidine binding studies. Thereafter, the coupling of the expressed receptor protein to a variety of signal transduction pathways was investigated. After cotransfection of CHO cells with pCMVhumH_2 and PUT626 a phleomycine-resistant clonal cell line (CHOhumH_2) was isolated that expressed 565 ± 35 fmol/mg protein binding sites with high affinity (0.21 ± 0.02 nM) for the H_2 antagonist [^{125}I]-iodoaminopotentidine. The encoded protein was indistinguishable from the H_2 receptor identified in human brain membranes and guinea-pig right atrium as the K_i-values of a variety of H_2 antagonists correlated well (r^2 = 0.996 and 0.920 respectively). Displacement studies with histamine showed that a limited fraction (32 ± 6%) of the binding sites showed a high affinity for histamine (2 ± 1.2 μM); the shallow displacement curves were reflected by a Hill-coefficient significantly different from unity (n_H = 0.58 ± 0.09). The addition of 100 μM Gpp(NH)p resulted in a steepening of the displacement curve (n_H = 0.79 ± 0.02) and a loss of high affinity sites for histamine. The recently developed specific H_2 agonists amthamine and amselamine showed an approximately 4-5 fold higher affinity for the human H_2 receptor than histamine.

Stimulation of CHOhumH_2 cells with histamine resulted in a rapid rise of the intracellular cAMP levels (EC_{50}-value 7 ± 1 nM) which was effectively blocked by tiotidine and cimetide (K_i-values of 8 ± 1 nM and 0.56 ± 0.24 μM respectively). Stimulation of CHOhumH_2 cells with histamine neither inhibited the A23187-induced release of [^{3}H]-arachidonic acid nor changed the intracellular IP_3 levels.

These results show that the cloned human gene encodes an histamine H_2 receptor that is indistinguishable from the H_2 receptor identified in human brain tissue. This receptor is functionally coupled to the adenylate cyclase in CHO cells, but does not influence the inositol phosphate turnover or arachidonic acid release.

Introduction

Following the original observations of Ash and Schild (1966) of possible histamine receptor heterogeneity, Black *et al.* (1972) initiated a successful search for histaminergic ligands that could selectively block or mimic the actions of histamine on cardiac and stomach function. It was the development of burimamide and various analogues (Black *et al.*, 1972) that finally provided evidence for the existence of at least two different histamine receptor subtypes. With the availability of suitable pharmacological tools (for a review see Leurs *et al.*, 1991) the role of the H_2 receptor in human (patho)physiology has been studied in detail. The receptor protein is present in e.g. various distinct human brain areas (Traiffort *et al.*, 1992a, Martinez-Mir *et al.*, 1993), human heart (Bristow *et al.*, 1982, Du *et al.*, 1993, Zerkowski *et al.*, 1993), human airway preparations (Barnes, 1992), human blood vessels (Keitoku *et al.*, 1990, Toda, 1990), human uterus (Martinez-Mir *et al.*, 1992) and human stomach (Bertaccini and Corruzi, 1992). As can be anticipated from this localization the H_2 receptor subtype has been identified as an important target for (potential) pharmacotherapy. Besides a role of H_2 receptor

antagonists in gastric acid secretion (Bertaccini and Corruzi, 1992) also application of cardioselective H_2 receptor agonists in congestive heart failure has been suggested (Felix *et al.*, 1991). Moreover, recently, a role for the H_2 receptor has been suggested in some neurological diseases (Deutsch *et al.*, 1993, Kaminsky *et al.*, 1990, Martinez-Mir *et al.*, 1993), indicating that also CNS-active H_2 ligands might have therapeutic potential. Due to these perspectives, the search for more potent and selective H_2 receptor agents still continues and has, for instance, recently resulted in the development of the new and highly specific H_2 receptor agonists amthamine and amselamine (Eriks *et al.*, 1991, Timmerman, 1992, Van der Goot *et al.*, 1994).

Despite the interesting therapeutic potential of the H_2 receptor, detailed studies of the human histamine H_2 receptor have been hampered by the lack of suitable model systems. Human tissue is not widely available for pharmacological studies and only a few human cell lines have been reported to contain histamine H_2 receptors (Arima *et al.*,1991, Emani *et al.*, 1983, Gespach *et al.*, 1985). Yet, the reported pharmacology of the histamine receptor present on these cells is not always clear (Burde *et al.*, 1989, Mitsuhashi *et al.*, 1991, Reyl-Desmars *et al.*, 1991, Seifert *et al.*, 1992). Only in human monocytic U937 cells pharmacologically clearly defined H_2 receptors are present, but the density of receptor sites is too low for detailed receptor binding studies (Smit *et al.*, 1994).

The recent introduction of molecular biology in the field of G-protein coupled receptor research has greatly improved the knowledge of molecular aspects of these receptor proteins. Following the cloning of the gene encoding the hamster beta$_2$ receptor by Dixon *et al.* (1986), a wide variety of genes encoding G-protein coupled receptors have been cloned by various techniques. Also in the field of histamine research the molecular biological approach has recently been successful. Using degenerated oligonucleotides based on conserved transmembrane regions of the receptor family and mRNA of canine parietal cells, Gantz *et al.* (1991a) succeeded in cloning the gene encoding a putative canine histamine H_2 receptor. Using the canine nucleotide sequence, soon thereafter a rat (Ruat *et al.*, 1991) and human homologue (Gantz *et al.*, 1991b) were cloned. The rat gene was expressed in Chinese Hamster Ovary cells and was shown to be a typical H_2 receptor (Traiffort *et al.*, 1992b). For the proteins encoded by the canine and human gene no detailed information is available yet for the pharmacological profile of the encoded proteins.

A cellular system expressing the human histamine H_2 receptor protein would be very useful for the development of new histaminergic ligands and would also allow detailed investigation of the regulation of the histamine H_2 receptor function. For the study of H_1 receptor regulation isolated cell systems have already proven to be extremely useful (see e.g. Smit *et al.*, 1992). In view of the anticipated use of histamine H_2 receptor agonists in congestive heart failure (Felix *et al.*, 1991) and the reported increase in beta-receptor kinase expression in heart tissue of patients with congestive heart failure (Ungerer *et al.*, 1993), a detailed study of the molecular mechanisms underlying the regulation of histamine H_2 receptor function is of great

importance. Therefore, we decided to stably express the reported human gene in Chinese Hamster Ovary cells and to perform a molecular pharmacological characterization of the encoded protein. Besides receptor binding studies with a wide range of histaminergic agents we also investigated the signal transduction pathways that can be activated by the receptor protein in these cells.

Materials and methods

Cell culture CHO cells deficient in dihydrofolate reductase were maintained at 37 °C in a humified atmosphere with 5% CO_2 in Dulbeco's Modified Eagle's Medium (DMEM), containing 10% (vol/vol) fetal calf serum and supplemented with 2 mM L-glutamine, hypoxanthine, thymidine, MEM amino acids, 50 IU/ml penicilline and 50 µg/ml streptomycine. CHO cells were stably transfected with the eukaryotic expression vector pCMVhumH_2neo (Gantz *et al.*, 1991b) and the plasmid pUT626, containing the *Sh ble* gene, conferring resistance to the antibiotic phleomycine (Cayla, France), using Lipofectine (Gibco BRL, The Netherlands). After two weeks of selection in the presence of 50 µg/ml phleomycine, surviving CHO colonies were isolated by ring cloning and further expanded in culture medium supplemented with phleomycine.

CHO cells expressing the guinea-pig histamine H_1 receptor (CHOgpH_1, Leurs *et al.*, 1994) were grown at 37°C in a humified atmosphere with 5% CO_2 in Dulbeco's Modified Eagle's Medium (DMEM), containing 10% (vol/vol) dialysed fetal calf serum and supplemented with 2 mM L-glutamine, MEM amino acids, 50 IU/ml penicilline and 50 µg/ml streptomycine.

Biochemical measurements The determinations of H_2 receptor binding and levels of cAMP were performed as described in Chapter 5.

[^{3}H]-Arachidonic acid release CHOhumH_2 cells were seeded in 24-well plates and cultured overnight in culture medium, containing 0.5 µCi/ml [^{3}H]-arachidonic acid ([^{3}H]-AA). To remove unincorporated [^{3}H]-AA cells were washed twice with 1 ml DMEM supplemented with 0.2% bovine serum albumin (fatty acid free). Thereafter the cells were incubated at 37°C for 30 mins with the appropriate drugs in 1 ml of DMEM with 0.2% bovine serum albumin. In some experiments CHOhumH_2 cells were preincubated with histamine for 10 mins before the release of [^{3}H]-AA was stimulated. The release of [^{3}H]-AA was determined by liquid scintillation counting of 0.5 ml sample of the incubation medium. Previously, it has been shown that over 90% of the released radioactivity corresponds to authentic [^{3}H]-AA (Traiffort *et al.*, 1992b).

Inositol phosphate production CHOhumH_2 or CHOgpH_1 cells were seeded in 24-well plates and cultured overnight in culture medium. Cells were washed twice with DMEM, supplemented with 50 mM HEPES (pH = 7.4 at 37°C) and preincubated for 30 mins at 37°C. Thereafter the medium was aspirated, 100 µM histamine in 100 µl DMEM/HEPES was added and the cells were incubated for the indicated time at 37°C. The reaction was stopped by the addition of 100 µl of 10% cold $HClO_4$. The cells were disrupted by sonication (5 sec, 50 Watt, Labsonic 1510, Braun-Melsungen) and kept on ice for 10 mins The cell homogenate was centrifuged for 5 min in Eppendorf tubes at 11.000 g. The supernatant (190 µl) was transfered to another tube and 47.5 µl 10 mM EDTA (pH = 7.4) was added. The samples were neutralized with 300 µl of a freon:tri-octylamine mixture (1:1, vol/vol, freshly prepared). The samples were vortexed and centrifuged for 2 mins at 11,000 g. The neutralized upper phase (150 µl) was mixed with 37.5 µl 1 M $NaHCO_3$ and assayed for the presence of IP_3 using a IP_3-mass assay system (Amersham).

In some experiments cells were labelled overnight in culture medium supplemented with 1 µCi/ml [^{3}H]-inositol. Reactions were terminated with 1 ml cold $CHCl_3$/methanol and the [^{3}H]-inositol phosphates were isolated by anion exchange chromatography (Godfrey, 1992).

Chemicals Histamine dihydrochloride, A23187, isobutylmethylxanthine, cyclic AMP, haloperidol and mepyramine were obtained from Sigma Chemical Company (USA). [^{3}H]-cAMP (40 Ci/mmol), [^{3}H]-inositol (18.8 Ci/mmol) were obtained from Amersham, whereas [^{3}H]-arachidonic acid (232 Ci/mmol) was purchased from New England Nuclear. Dimaprit dihydrobromide, amthamine dihydrobromide, amselamine dihydrobromide, impromidine trihydrobromide, aminopotentidine and thioperamide were taken from laboratory stock. Gifts of burimamide, metiamide, cimetidine, icotidine, zolantidine (SmithKline Beecham), tiotidine (ICI), ranitidine (Glaxo), famotidine (MSD), mianserin (Organon) and the human H_2 cDNA (Dr. I. Gantz) are acknowledged.

Results

Pharmacological characterization of the human H_2 receptor using [^{125}I]-APT

Cotransfection of CHO cells with the plasmid pCMVhumH_2 and pUT626 resulted after 10-12 days of selection in culture medium supplemented with 50 μg/ml phleomycine in the formation of several clonal cell lines, which expressed [^{125}I]-APT binding sites. Untransfected cells did not show any specific [^{125}I]-APT binding (data not shown). For subsequent experiments a cell line (CHOhumH_2) stably expressing [^{125}I]-APT binding sites was further analysed. Saturation experiments performed with CHOhumH_2 cell membranes indicated the expression of a single [^{125}I]-APT binding site (n_H = 1.0 ± 0.1) with a dissociation constant (K_d) of 0.21 ± 0.02 nM (n = 3, mean ± s.e.mean) and a maximal density of 565 ± 35 fmol/mg protein (n = 3, mean ± s.e.mean) (Fig. 1).

The specific binding of 0.2 nM [^{125}I]-APT represented more than 95% of the total binding and was monophasically (n_H not significantly different from unity) inhibited by a variety of H_2 receptor antagonists but was unaffected by the H_1 and H_3 antagonists mepyramine and thioperamide (Fig. 2A, Table 1). The K_i-values of the H_2 antagonists correlated well with their biological activities at the H_2 receptor of the guinea-pig right atrium (Leurs *et al.*, 1991) (r^2 = 0.920, n = 10, Table 1) and the K_i-values determined for the H_2 receptor on *post-mortem* human brain membranes (Traiffort *et al.*, 1992a) (r^2 = 0.996, n = 6, Table 1). The antidepressant mianserine and the neuroleptic haloperidol also interacted with the cloned human H_2 receptor protein, although at only moderate concentrations (Table 1).

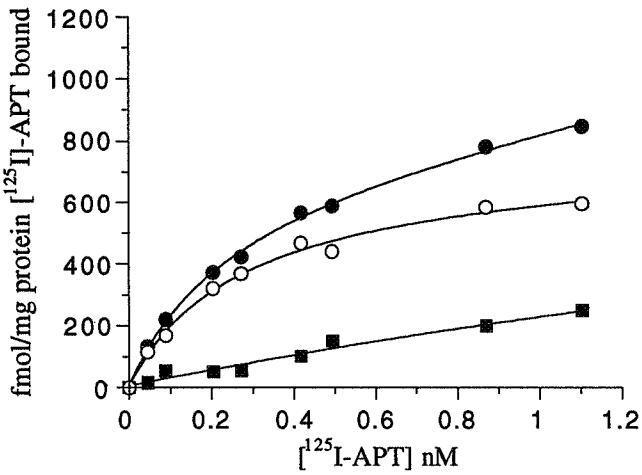

Fig. 1. Concentration dependent binding of [^{125}I]-APT to CHOhumH_2 cell membranes. Specific radioligand binding (open circles) was determined by substracting the binding in the presence of 1 μM of tiotidine (filled squares) from the total binding (filled circles). Mean values of triplicate determinations of a typical experiment out of three are shown.

Table 1 K_i-values of various histaminergic antagonists for competition of [^{125}I]-APT binding to CHOHumH$_2$ membranes

	CHOHumH$_2$		human brain	guinea-pig atrium
compounds	K_i	n_H	K_i	K_B
APT	15 ± 5 nM	-0.99 ± 0.6	-	-
Burimamide	3.9 ± 1.2 μM	-1.10 ± 0.04	1.6 μM	7.8 μM
Cimetidine	0.66 ± 0.22 μM	-0.89 ± 0.02	0.27 μM	0.79 μM
Famotidine	16 ± 3 nM	-0.92 ± 0.07	-	16 nM
Metiamide	0.67 ± 0.16 μM	-0.95 ± 0.03	-	0.92 μM
Ranitidine	85 ± 4 nM	-0.96 ± 0.06	38 nM	63 nM
Tiotidine	17 ± 6 nM	-0.88 ± 0.01	5 nM	15 nM
Zolantidine	40 ± 8 nM	-1.05 ± 0.06	-	35 nM
Icotidine	15 ± 2 nM	-1.01 ± 0.04	-	60 nM
Haloperidol	1.5 ± 0.3 μM	-0.98 ± 0.03	0.65 μM	0.87 μM
Mianserine	0.56 ± 0.51 μM	-1.08 ± 0.04	0.10 μM	0.11 μM
Mepyramine	> 10 μM			
Thioperamide	> 100 μM			

Reported values for [^{125}I]-APT binding to human brain membranes (Traiffort *et al.*, 1991a) and antagonism of histamine-induced chronotropic responses of guinea-pig right atrium (Leurs *et al.*, 1991) are shown for comparison. Data shown are mean ± s.e.mean of three to four independent experiments, each performed in triplicate.

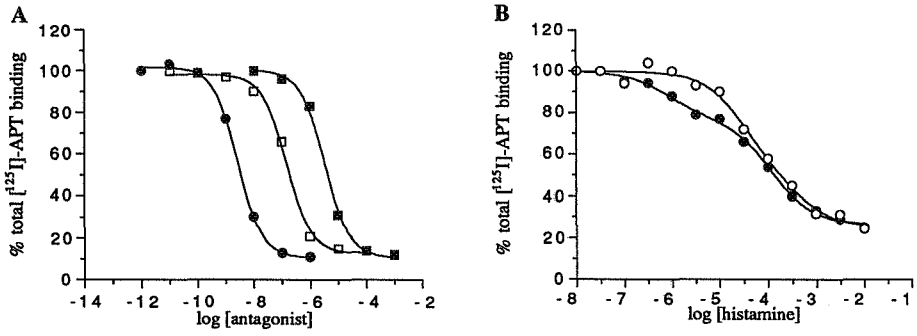

Fig. 2. Pharmacological profile of the [^{125}I]APT binding site on CHOhumH$_2$ cell membranes. Displacement of [^{125}I]-APT binding by (A) APT (filled circles), ranitidine (open circles), burimamide (filled squares) or (B) histamine in the absence (filled circles) or presence (open circles) of 100 μM of Gpp(NH)p is shown. Data shown are the mean values of triplicate determinations from a typical experiment. Similar results were obtained in 2-4 other independent experiments.

Table 2 K_i-values of various histaminergic agonists for competition of [^{125}I]-APT binding to CHOHumH$_2$ membranes in the absence or presence of 100 μM Gpp(NH)p. Reported values for agonist-induced chronotropic responses of guinea-pig right atrium (Eriks *et al.*, 1991, Van der Goot, 1994) are shown for comparison. Values between parentheses indicate the relative densities of the respective sites. Data shown are mean ± SEM of three to five independent experiments, each performed in triplicate

compounds	- Gpp(NH)p			+ Gpp(NH)p		guinea-pig right atrium
	$K_{i,\ high}$ (μM)	$K_{i,\ low}$ (μM)	n_H	K_i (μM)	n_H	EC_{50} (μM)
Histamine	2.0 ± 1.2 (32 ± 6%)	81 ± 15 (68 ± 6%)	-0.58 ± 0.09	45 ± 4	-0.79 ± 0.02	0.72
Amthamine	0.15 ± 0.14 (23 ± 6%)	6.9 ± 0.8 (76 ± 6%)	-0.68 ± 0.03	7.0 ± 1.7	-0.88 ± 0.01	0.62
Amselamine	0.13 ± 0.09 (18 ± 3%)	9.6 ± 0.7 (82 ± 3%)	-0.73 ± 0.02	10 ± 1	-1.02 ± 0.11	0.39
Dimaprit	ND	25 ± 5 (100%)	-0.79 ± 0.02	24 ± 5	-0.96 ± 0.09	1.1
Impromidine	0.023 ± 0.028 (31 ± 15%)	0.38 ± 0.09 (69 ± 16%)	-0.73 ± 0.04	0.47 ± 0.03	-0.95 ± 0.04	0.016

ND = not detectable

Various histamine H_2 agonists displaced the specific [^{125}I]-APT binding to CHOhum H_2 cell membranes too (Fig. 2B, Table 2). Yet, histamine displacement curves were shallow (n_H = 0.58 ± 0.09, n = 5, mean ± s.e.mean) and could be best analysed according to a two site model (Fig. 2B). Simultaneous fitting of the five independent experiments resulted in an affinity of 2.0 ± 1.2 μM and 81 ± 15 μM for respectively the high- and low affinity binding site. The addition of 100 μM Gpp(NH)p resulted in a steepening and a rightward shift of the histamine displacement curve (Fig. 2B), which was best analysed according to a single site model , leading to a K_i-value of 45 ± 4 μM (Table 2). The percentage high affinity sites for histamine was rather low (Fig. 2B, Table 2) and not always obvious. Similar observations were made for the other H_2 receptor agonists. Under control conditions shallow displacements curves were obtained, which, with the exception of the data for dimaprit, were best fitted according to a two sites model (Table 2). In the presence of Gpp(NH)p the specific agonists amthamine (Eriks *et al.*, 1991), amselamine (Van der Goot *et al.*, 1994), dimaprit and impromidine all displaced [^{125}I]-APT binding to CHOhumH_2 membranes monophasically and showed a higher affinity for the human H_2 receptor compared to histamine (Table 2).

cAMP production in CHOhumH_2 cells

In untransfected CHO cells histamine (100 μM) did not affect the cellular cAMP level (data not shown). Stimulation of the CHOhumH_2 cells in the absence of the cAMP phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) with 1 μM histamine for 10 mins resulted in a slight but significant increase of the basal cAMP level of 6.6 ± 1.0 pmol/well to 10.7 ± 1.1 pmol/well ($p < 0.05$, mean ± s.e.mean, n = 3). In the presence of increasing concentrations of IBMX, histamine stimulation resulted in a massive increase of the cellular cAMP levels, which appeared to be maximal with 300-600 μM IBMX (data not shown). In subsequent experiments the cAMP accumulation was measured in the presence of 300 μM IBMX.

Under these conditions addition of 1 μM histamine resulted in the rapid production of cellular cAMP (inset Fig. 3A). Already 2 mins after the application of histamine, increased levels of cAMP were found. Maximal stimulation of the cellular cAMP levels (approximately 10-fold) was observed after 10 mins (inset Fig. 3A). Stimulation of CHOhumH_2 cells with increasing concentrations of histamine for 10 mins resulted in a concentration dependent increase in cellular cAMP levels. At relatively low concentrations histamine stimulated the cAMP production (Fig. 3A); the EC_{50}-value of histamine for this response was 7 ± 1 nM (n = 5, mean ± s.e.mean). This histamine-induced (0.3 μM) cAMP response was effectively inhibited by the H_2 receptor antagonists tiotidine and cimetidine (Fig. 3B), resulting in K_i-values of 8 ± 1 nM and 0.56 ± 0.24 μM (n = 4, mean ± s.e.mean) respectively. These K_i-values correspond well with the K_i-values obtained from the [^{125}I]-APT displacement studies (Table 1). The histamine-induced cAMP response was not affected by the H_1 and H_3 receptor antagonists mepyramine and thioperamide in concentrations up to 100 μM (Fig. 3B).

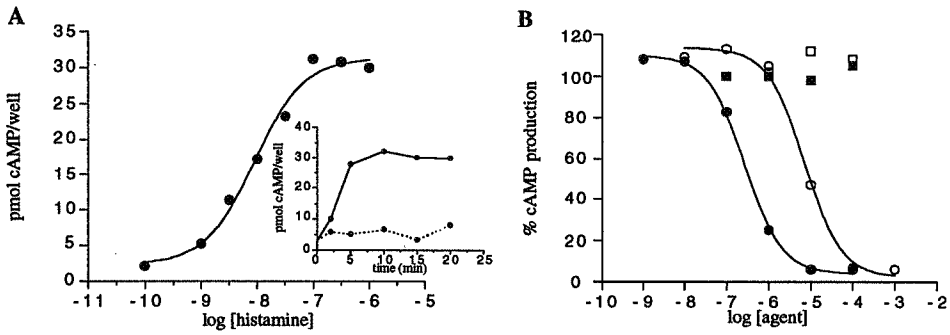

Fig. 3. Histamine-induced cAMP accumulation in CHOhumH$_2$ cells. (A) CHOhumH$_2$ cells were stimulated with increasing concentrations of histamine for 10 mins at 37°C in the presence of 300 μM of IBMX. Data shown are the mean values of a typical experiment out of 6. In untransfected cells histamine did not alter the intracellular cAMP concentration. Inset: CHOhumH$_2$ cells were stimulated with DMEM (dotted line) or 1 μM of histamine (solid line) for various lengths of time at 37°C in the presence of 300 μM of IBMX. Data shown are the mean values of a typical experiment out of 3.(B) Pharmacological characterization of the histamine-induced cAMP response in CHOhumH$_2$ cells. Cells were incubated for 10 mins at 37°C with 0.3 μM of histamine in the presence of increasing concentrations of tiotidine (filled circles), cimetidine (open circles), mepyramine (filled squares) and thioperamide (open squares) and 300 μM of IBMX. A typical experiment out of 4 is shown.

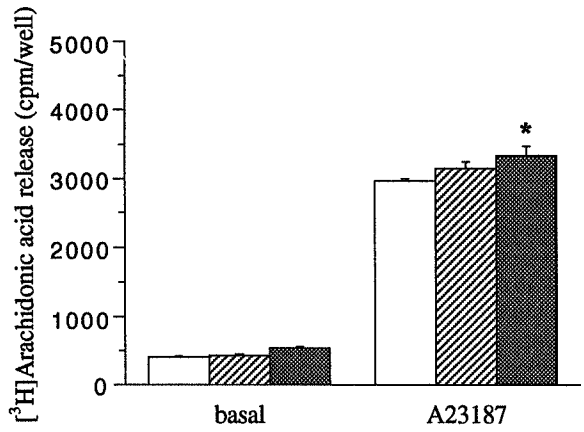

Fig. 4. Effect of histamine on the basal and A23187-induced release of [^{3}H]-AA from prelabelled CHOhumH$_2$ cells. Cells were incubated with DMEM (open column), 1 μM (hatched column) or 100 μM (filled column) of histamine in the absence or presence of 1 μM of A23187 for 30 mins at 37°C, whereafter the released radioactivity was counted in the medium. A typical experiment out of 4 is shown. * indicates a significant difference compared to stimulation with DMEM.

Arachidonic acid release in CHOhumH_2 cells

In CHOhumH_2 cells prelabelled with [^{3}H]-AA application of the Ca^{2+} ionophore A23187 led to a massive increase of [^{3}H]-AA release (Fig. 4). Whereas under basal conditions the [^{3}H]-AA release amounted to 389 ± 17 dpm (n = 4, mean ± s.e.mean), stimulation of CHOhumH_2 cells with 1 μM of the Ca^{2+} ionophore A23187 led to an elevation of the [^{3}H]-AA release to 3156 ± 88 dpm (n = 4, mean ± s.e.mean). Histamine (1 μM and 100 μM) did not affect the basal [^{3}H]-AA release, whereas the A23187 (1 μM) induced [^{3}H]-AA release was slightly increased by 100 μM of histamine (Fig. 4). In some experiments CHOhumH_2 cells were preincubated with histamine in order to study its effect on the A23187-induced [^{3}H]-AA release. Also under these conditions histamine had no inhibitory effect on the A23187 response (data not shown).

Inositol phosphate production in CHOhumH_2 cells

In CHOhumH_2 cells prelabelled overnight with [^{3}H]-inositol application of 100 μM histamine in the presence of 20 mM LiCl for 10 mins at 37°C did not result in an increase of the intracellular accumulation of [^{3}H]-inositol phosphates (107 ± 7% of the basal accumulation, mean ± s.e.mean, n = 3). Yet, stimulation of the endogenous P_2-purinergic receptor of CHOhumH_2 cells (Iredale and Hill, 1993, Traiffort *et al.*,1992b) with 100 μM of ATP resulted in an increase of 580 ± 110% (mean ± s.e.mean, n = 3) of the basal release of [^{3}H]-inositol phosphates. Since this approach might be too insensitive for small increases in the levels of inositol-1,4,5-trisphosphate (IP_3), we analysed the effects of histamine in more detail. Using an IP_3 mass assay we measured the intracellular IP_3 levels in CHOhumH_2 and CHOgpH_1 cells after stimulation with 100 μM histamine. As can be seen in Fig. 5, histamine had no effect on the basal levels of IP_3 in CHOhumH_2 cells. In CHOgpH_1 cells 100 μM histamine rapidly elevated, as expected, the cellular IP_3 levels; within 10 secs the IP_3 levels were more than doubled. This rise was transient and dropped to a lower level, which remained elevated up to 1 min of stimulation (Fig. 5). In accordance with these findings we were unable to find any effect of histamine on the intracellular calcium levels in fura-2 loaded CHOhumH_2 cells, although in the same population of cells 100 μM of ATP resulted in a rapid rise of the basal level of 97 ± 12 nM to a peak value of 406 ± 55 nM (mean ± s.e.mean, n = 3).

Discussion

In this paper we present evidence that the receptor protein encoded by the putative human histamine H_2 receptor gene (Gantz *et al.*, 1991b) is indistinguishable from the pharmacologically characterized H_2 receptor at the guinea-pig atrium (Leurs *et al.*, 1991) or human brain tissue (Traiffort *et al.*, 1992a). In the initial paper on the cloning of the human gene (Gantz *et al.*, 1991b), some indications of the H_2 nature of the encoded receptor protein were presented; transfected cells bound the H_2 receptor antagonist [^{3}H]-tiotidine, whereas

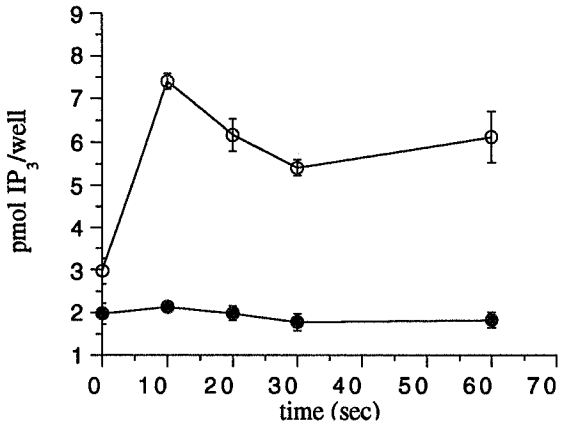

Fig. 5. Effect of 100 μM of histamine on the IP_3 production in $CHOhumH_2$ (filled circles) and $CHOgpH_1$ cells (open circles). Cells were incubated for the indicated times with histamine at 37°C, homogenized and the IP_3 levels were determined by radioreceptor assay. Data shown are the mean ± s.e.mean of a typical experiment out of 4.

histamine was found to induce a cimetidine-sensitive cAMP accumulation (Gantz *et al.*, 1991b). In view of the reported pharmacological discrepancies of human H_2 receptor-like proteins in various cell systems (Burde *et al.*, 1989, Reyl-Desmars *et al.*, 1991, Seifert *et al.*, 1992) the observed actions of cimetidine and tiotidine do, however, not unambiguously define the encoded human receptor protein.

In human gastric HGT-1 tumour cells Reyl-Desmars *et al.* (1991) reported the presence of an H_2 receptor with a rather low affinity for tiotidine. The pharmacological profile of the reported binding site was therefore quite different from the profile of standard H_2 receptor systems (Reyl-Desmars *et al.*, 1991) and still remains undefined. Nevertheless, functional H_2 receptors have been observed in the same cell line (Emani *et al.*, 1983). Similar observations are made in human HL-60 promyelocytic leukemia cells. Also in these cells functional H_2 receptors have been described (Burde *et al.*, 1989, Mitsuhashi *et al.*, 1991, Seifert *et al.*, 1992). Yet, the pharmacological definition of the various functional responses is not always clear. A unique agonist/antagonist profile is reported for the inhibition of the oxidative burst (Burde *et al.*, 1989) and the increase of cytosolic calcium in HL-60 cells (Mitsuhashi *et al.*, 1991, Seifert *et al.*, 1992).

In view of these data and the lack of suitable systems for the study of the pharmacological properties of the human H_2 receptor and the regulation of its fuction, we stably expressed the cloned human gene (Gantz *et al.*, 1991b) in CHO cells. Our detailed pharmacological characterization of the encoded protein was performed with the highly specific H_2 receptor

antagonist [^{125}I]-iodoaminopotentidine (Ruat *et al.*, 1990). This radiolabel has previously successfully been used for the labelling of the human H_2 receptor in brain tissue (Traiffort *et al.*, 1992a) and shows many advantages over [^{3}H]-tiotidine. From our results with CHOhumH_2 cells the H_2 nature of the encoded receptor protein is clear. Affinity constants calculated from displacement studies with 11 structurally different H_2 receptor antagonists perfectly match the pharmacological profile of the H_2 receptor from guinea-pig right atrium (Leurs *et al.*, 1991) and human brain (Traiffort *et al.*, 1992a); regression coefficients of 0.92 and 0.99 respectively are obtained when the various K_i-values are correlated. It can therefore be concluded that the cloned gene (Gantz *et al.*, 1991b) encodes a classical human H_2 receptor.

In this study a complicated interaction of histamine with the [^{125}I]-iodoaminopotentidine ([^{125}I]-APT) binding site on CHOhumH_2 cell membranes is observed. Shallow displacement curves with Hill-coefficients of 0.58 were obtained, whereas the inclusion of the non-hydrolysable GTP analogue Gpp(NH)p resulted in a steepening of the curves. Approximately 30% of the [^{125}I]-APT binding sites showed high affinity for histamine (2 μM). The biphasic nature of the displacement curves of histamine indicate the coupling of the human H_2 receptor to G-proteins.

As far as we know, the coupling of the human H_2 receptor to a G-protein has not been reported before on the basis of radioligand binding studies. Yet, besides its deduced amino acid structure, adenylate cyclase studies with human heart tissue (Bristow *et al.*, 1982) and radioligand binding studies with the rat H_2 receptor expressed in CHO cells (Traiffort *et al.*, 1992b) also indicate the interaction of the H_2 receptor with G-proteins. Remarkable is the discrepancy between the results from the displacement studies with histamine using CHOhumH_2 cell membranes and *post-mortem* human brain membranes (Table 1). In human brain tissue histamine was found to display a 10-fold higher affinity (4 μM) compared to guinea-pig brain tissue (Traiffort *et al.*, 1992a). The affinity of histamine for the high affinity site on CHOhumH_2 cell membranes is remarkably close to the reported affinity of histamine on human brain membranes (Traiffort *et al.*, 1992a). Unfortunately no data regarding GTP sensitivity were reported in the latter. Yet, the observed differences between guinea-pig and human brain tissue were suggested to be related to species differences or *post-mortem* or aging-induced changes in receptor characteristics (Traiffort *et al.*, 1992a). Based on the present findings we conclude that the previously reported high affinity of histamine in human brain membranes does not reflect a natural higher sensitivity of the human H_2 receptor to this neurotransmitter, but could be due to either conservation problems inherent to the use of *post-mortem* human samples or due to aging related changes in receptor or G protein characteristics; it is possible that only high affinity H_2 receptor sites have been measured in the *post-mortem* human brain membranes (Traiffort *et al.*, 1992a).

Following the characterization of the binding characteristics of the expressed protein we investigated the signal transduction pathways of the human H_2 receptor in CHO cells.

Previously, it has been reported that H_2 receptors can elevate the cAMP levels in a variety of human tissues (Arima *et al.*, 1991, Bristow *et al.*, 1982, 1989, Emani *et al.*, 1983, Platshon *et al.*, 1978, Whitehead *et al.*, 1988). We found that also in stably expressed CHO cells stimulation of the human H_2 receptor results in the rapid intracellular accumulation of cAMP. Histamine is highly effective in this respect; the high potency in relation to its low affinity reflects a large receptor reserve for histamine and therefore a highly efficient coupling of the H_2 receptor to the adenylate cyclase system in the CHO cells. The histamine-induced cAMP production in CHOhumH_2 cells was pharmacologically characterized as an H_2 receptor mediated process; the response was not inhibited by the H_1 and H_3 receptor antagonists mepyramine and thioperamide but could potently be inhibited by the H_2 antagonists tiotidine and cimetidine. The resulting K_i-values obtained from the cAMP inhibition experiments (8 nM and 0.56 μM) were quite similar to the K_i-values obtained from the radioligand binding studies (17 nM and 0.66 μM) for tiotidine and cimetidine respectively.

In some systems histamine H_2 receptor stimulation has been suggested to be linked to other signal transduction mechanisms (Delvalle *et al.*, 1992, Mitsuhashi *et al.*, 1989, Seifert *et al.*, 1992, Traiffort *et al.*, 1992b). In HL-60 cells (Mitsuhashi *et al.*, 1989, Seifert *et al.*, 1992) and rat HEPA cells transfected with the canine H_2 receptor cDNA (Delvalle *et al.*, 1992) H_2 receptor stimulation has been reported to result in an increase of the intracellular Ca^{2+} concentration. In HEPA cells this response was clearly correlated with an increase of the IP_3 levels and appeared to be independent from the cAMP production (Delvalle *et al.*, 1992). Moreover, in CHO cells rat H_2 receptor stimulation inhibited A23187-induced arachidonic acid release via a cAMP independent mechanism (Traiffort *et al.*, 1992b). These data indicate that H_2 receptors might interact with a variety of different G proteins in the membrane.

In CHOhumH_2 cells we were unable to observe a coupling to the phospholipase C and phospholipase A_2 pathway. In total agreement with our findings a linkage of the rat H_2 receptor with the phospholipase C pathway could not be found either (Traiffort *et al.*, 1992b). These conflicting observations can be explained by assuming different G protein and/or phospholipase C isoenzyme distribution in CHO and HEPA cells. In contrast to the rat H_2 receptor (Traiffort *et al.*, 1992b) the human H_2 receptor was not able to reduce the A23187-induced arachidonic acid release from CHOhumH_2 cells either. Instead, a slight stimulation was obtained, which can be explained by the reported modulation of arachidonic acid release from CHO cells by protein kinase A (Piomelli and Di Marzo, 1993). An explanation for the observed lack of inhibition could be found in the difference in receptor expression. For the rat H_2 receptor a CHO clone expressing 1.4 pmol/mg protein was used for the arachidonic acid release experiments (Traiffort *et al.*, 1992b), whereas in the present study the human H_2 receptor was expressed at a more physiological level (± 600 fmol/mg protein). It is also possible that the observed sequence differences between the rat (Ruat *et al.*, 1991) and human gene (Gantz *et al.*, 1991) are responsible for the difference in signal transduction. It is known that intracellular receptor domains are involved in the interaction of G protein coupled receptors

with different G proteins (Probst *et al.*, 1992). Although the rat and human H_2 receptor genes share a high degree of overall homology, especially in the intracellular parts of the receptor proteins differences are found (Gantz *et al.*, 1991b, Ruat *et al.*, 1991).

In conclusion, we have stably expressed the human H_2 receptor in CHO cells. The receptor protein is pharmacologically similar to the human H_2 receptor in brain tissue and is very efficiently coupled to the adenylate cyclase system. The availability of a cell system expressing the human H_2 receptor will play a key role in the development of new and selective ligands for this protein, an important target for today's human pharmacotherapy (Bertaccini and Corruzi, 1992, Felix *et al.*, 1991). Screening of large series of compounds for their affinities for the human H_2 receptor is possible now, whereas also the regulation of the human H_2 receptor function can be studied in detail.

H_2 receptor agonists are investigated for future development for application in patients with congestive heart failure (Felix *et al.*, 1991). In this pathophysiological condition the cardiac adrenergic neurotransmission is strongly reduced. This is probably the result of an increase in the expression of the beta-adrenergic receptor kinase, which is responsible for the phosphorylation and desensitization of the $beta_2$ adrenoceptor (Ungerer *et al.*, 1993). Recently, we observed a rapid desensitization of the human H_2 receptor-mediated cAMP production in U-937 cells (Smit *et al.*, 1994). Yet, the use of this cell line for detailed biochemical investigations is limited since the density of H_2 receptors is very low (±10 fmol/mg protein, M.J. Smit, unpublished observations). The use of the described CHOhumH_2 cells will therefore be also of great importance for future mechanistic studies of the desensitization and regulation of H_2 receptor expression.

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Chapter 8

Visualization of agonist-induced internalization of histamine H_2 receptors

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Histamine H_2 receptors were tagged at the N-terminus with the eight amino acid Flag epitope to allow the immunological identification of the receptor peptide with the monoclonal anti-Flag M2 antibody. The introduction of the epitope did not modify the binding of several H_2 ligands to the H_2 receptor, nor the ability of histamine to stimulate the H_2 receptor mediated cAMP production in HEK-293 cells. Western blots revealed a major protein band of 57 ± 1 kDa, whereas a second band of 31 ± 1 kDa was probably the result of a proteolytic breakdown of the 57 kDa band. Immunofluorescence measurements of stably transfected HEK-293 cells revealed the presence of anti-Flag-immunoreactivity in the plasma membrane. This immunoreactivity completely disappeared after a one hour treatment with histamine. The receptor internalization was reversible and blocked by the endocytosis inhibitor phenylarsine oxide. Forskolin did not induce H_2 receptor internalization, indicating that histamine causes H_2 receptor internalization via a cAMP-independent pathway.

Introduction

Molecular biological studies indicate that the histamine H_2 receptor belongs to the large multigene family of G protein coupled receptors (GPCR) (Gantz *et al.*, 1991, Gantz *et al.*, 1991, Ruat *et al.*, 1991, Traiffort *et al.*, 1995). Excessive stimulation of receptor proteins belonging to the GPCR family often leads to an attenuation of the receptor responsiveness (Collins, 1993, Lohse, 1993). Three major mechanisms are currently acknowledged to be involved in the process of GPCR regulation (Collins, 1993, Lohse, 1993). Initially, GPCRs are uncoupled from their respective G proteins due to phosphorylation by serine/threonine kinases, resulting in a desensitization of the receptor responsiveness (Collins, 1993, Lohse, 1993). Thereafter, GPCRs can be translocated from the plasma membranes to intracellular endosomes, where they are inaccessible to hydrophylic ligands like biogenic amines (Collins, 1993, Lohse, 1993). Finally, the GPCRs can be degradated to obtain a lower expression level at the plasma membrane for a prolonged period of time (Collins, 1993, Lohse, 1993).

Despite the wide therapeutic use of H_2 antagonists for gastric ulcers (Bertaccini and Coruzzi, 1992) and the potential application of H_2 agonists in cardiac heart failure (Felix *et al.*, 1991), only limited information is available on the regulation of the H_2 receptor responsiveness. Previously, we and others showed that the H_2 receptor mediated cAMP response is rapidly desensitized in various cell types (Arima *et al.*, 1993, Fukushima *et al.*, 1994, Johnson and Sawutz, 1984, Prost *et al.*, 1984, Schreurs *et al.*, 1984, Smit *et al.*, 1994). With the availability of the nucleotide sequence encoding the H_2 receptor (Gantz *et al.*, 1991, Gantz *et al.*, 1991, Ruat *et al.*, 1991, Traiffort *et al.*, 1995) detailed molecular investigations of H_2 receptor regulation are now feasible. Cellular systems, expressing

sufficient amounts of H_2 receptor proteins for the detection with radioligands, have been reported (Fukushima *et al.*, 1994, Leurs *et al.*, 1994, Traiffort *et al.*, 1992) and shown to be highly suitable for the study of H_2 receptor regulation.

We previously reported on the agonist-induced H_2 receptor downregulation in CHO cells, expressing the rat histamine H_2 receptor (Chapter 5). Our data indicate that the general scheme of regulation of GPCR responsiveness is probably also applicable to the H_2 receptor. Currently, no studies on H_2 receptor internalization have been reported. This is mainly due to the lack of suitable hydrophylic radioligands and anti-H_2 receptor antibodies. Again, the cloning of the H_2 receptor cDNAs and genes (Gantz *et al.*, 1991, Gantz *et al.*, 1991, Ruat *et al.*, 1991, Traiffort *et al.*, 1995) has provided means to overcome this latter problem. Recently, an anti-H_2 receptor peptide antibody has been described (Fukushima *et al.*, 1994), whereas in the present study we describe the construction of epitope-tagged H_2 receptors as a tool to identify the H_2 receptor protein by Western blotting and immunofluorescence microscopy. This approach allows us to demonstrate the process of H_2 receptor internalization for the first time.

Materials and Methods

Epitope-tagging H_2 receptor The nucleotide sequence encoding the epitope-tagged human H_2 receptor was constructed by PCR, using the synthetic oligonucleotide 5'-TGCAGATCTGCCACCATGGACTACAA-GGACGACGATGACAAGGCACCCAATGGCACAGCCTCT (nucleotides 4 to 24 of the human H_2 receptor (Gantz *et al.*, 1991), a nucleotide sequence encoding the Flag peptide DYKDDDD, a methionine, a Kozak sequence and a *Bgl*II linker site) and the oligonucleotide 5'-TGCAGATCTTTACCTGTCTGTGGCTCCCT (nucleotides 1061-1080 and a *Bgl*II linker site). For the rat H_2 receptor similar oligonucleotides 5'-GGGAAGCTTGCCACCATGGACTACAAGGACGACGATGACAAGGAGCCCAATGGCACAGTTCAT and 5'-CGGAGATCTACAACCTTTACCTGATTGGGT were used. Using 100 ng pSVratH_2 (Ruat *et al.*, 1991) or pCMVhumH_2 (Gantz *et al.*, 1991) as template, 0.4 µM of the respective oligonucleotides, 200 µM of each nucleotide and 2.5 U *Pfu* DNA polymerase (Promega), the desired fragments were amplified in 100 µl using 25 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min and a final extension at 72°C for 10 min. The DNA fragments were gel-purified, restricted with the appropriate enzymes and ligated in the plasmid pSP73 (Promega). The complete nucleotide sequence of the receptor mutant was verified using Sequenase (USB) and subcloned into the expression vector pRK_5.

Cell culture and transfection Human embryonic kidney cells (HEK-293 cells) were grown at 37°C in a humified atmosphere with 5% CO_2 in Dulbeco's Modified Eagle's Medium (DMEM), containing 10% (v/v) foetal calf serum and supplemented with 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. HEK-293 cells were transiently transfected with the eukaryotic expression vectors pRK_5, pRK_5humH_2, pRK_5humH_2Flag and pRK_5ratH_2Flag using calcium phosphate precipitation (Chen and Okayama, 1987). HEK-293 cells (10 cm dish) were stably cotransfected with the eukaryotic expression vectors pRK_5ratH_2Flag (14 µg) and pSV_2neo (1 µg) using Transfectam (Promega) (Leurs *et al.*, 1994). Stable cell lines were maintained in culture medium, supplemented with 500 µg/ ml G418.

Biochemical measurements The determination of the cAMP levels and the H_2 receptor binding assays were performed as described previously (Leurs *et al.*, 1994). In some experiments membrane proteins were solubilized (Ruat *et al.*, 1990) and resolved by electophoresis through a 10% polyacrylamide/SDS gel and transferred to nitrocellulose (BA85, Schleicher and Schuell). Immunoblotting was performed overnight at 4 °C with the anti-Flag M2 antibody (0.6 µg/ml) and an incubation with a horseradish peroxidase conjugated goat-anti-mouse secondary antibody (Biorad, 1:20.000). Immunoreactivity was visualized by chemiluminescence using the Amersham ECL detection kit.

Immunofluorescence microscopy HEK-293 cells were grown on glass coverslips. Following the various

treatments cells were fixed with 4% paraformaldehyde/PBS for 30 mins at room temperature (RT) and blocked in PBS/0.1% BSA for 1hr at RT. For detection of immunofluorescence inside the cell, cells were permeabilzed using 0.5% Nonidet P40 (Boehringer)/PBS for 30 mins at RT and blocked with PBS/0.1%BSA/0.4% Tritonx100. Receptor antigen detection was performed with 9 µg/ml anti-Flag M2 antibody for 2 hrs in PBS/1% BSA. After washing in PBS the coverslips were incubated with a fluorescein isothiocyanate-conjugated rabbit-anti-mouse secondary antibody (Dakopatt 1:100) for 1 hr in PBS/1% BSA. Immunofluorescence microscopy was performed with an inverted (Axiovert 135) Zeiss 410 confocal laser scan microscope. The standard filter combination for FITC fluorescence microscopy (FT 510, BP 515-565) was used. The FITC was excited using the 488 line of an Argon laser. The parameters of the microscope (laser intensity, pinhole, scantime, contrast, brightness) were kept constant during the experiments, allowing to compare fluorescence levels of cells during the different experimental conditions.

Chemicals Histamine.2HCl, forskolin, phenylarsine oxide, isobutylmethylxanthine were obtained from Sigma Chemical Company (USA). [^{3}H]-cAMP (40 Ci/mmol) was obtained from Amersham. Aminopotentidine was taken from laboratory stock. Generous gifts of tiotidine (Imperial Chemical Industries) and burimamide (SmithKline Beecham) are greatly acknowledged. The mouse anti-Flag M2 monoclonal antibody was obtained from International Biotechnology Inc.

Results

Transient expression of the human and rat histamine H_2Flag receptor in HEK-293 cells

Forty-eight hrs after transient transfection of HEK-293 cells with the expression vectors pRK_5humH_2 and pRK_5humH_2Flag a high expression of the respective H_2 receptor proteins was detected with the H_2 receptor antagonist [^{125}I]-iodoaminopotentidine ([^{125}I]-APT). The eight amino acid epitope had no effects on either the receptor expression levels ($humH_2$: B_{max} = 3.2 ± 0.7 pmol/mg protein, $humH_2Flag$: B_{max} = 2.8 ± 0.4 pmol/mg protein) or the K_d value for [^{125}I]-APT (Table 1). Moreover, the K_i values for other H_2 antagonists and histamine did also not markedly differ between the two receptor proteins (Table 1).

The Flag epitope did not affect the signalling properties of the receptor protein in transiently transfected HEK-293 cells either. Stimulation of HEK-293 cells expressing the human H_2Flag receptor with 10 nM or 1 µM histamine induced respectively 98 ± 2 % (n = 4) and 105 ± 8 % (n = 4) of the cAMP accumulation in HEK-293 cells expressing the wild-type receptor induced by the same concentrations of histamine.

Table 1 Analysis of the binding of several H_2 ligands to membranes of HEK-293 cells expressing the human wild-type or H_2Flag receptor protein. Data shown are mean ± s.e.mean of three independent experiments.

	K_i values	
compound	wild-type H_2	H_2Flag
[^{125}I]-APT*	0.34 ± 0.03 nM	0.35 ± 0.07 nM
aminopotentidine	12 ± 2 nM	9 ± 2 nM
tiotidine	14 ± 3 nM	12 ± 2 nM
burimamide	21 ± 5 µM	22 ± 10 µM
histamine	89 ± 5 µM	181 ± 19 µM

* K_d value

The Flag epitope allowed the detection of the human and rat H_2Flag receptor by the mouse anti-Flag M2 monoclonal antibody. Western blots of a 10 % SDS-PAGE gel of solubilized membranes of HEK-293 cells transiently transfected with pRK_5, pRK_5humH_2 or pRK_5humH_2Flag revealed only immunoreactive bands with cells, expressing the human H_2Flag receptor (Fig. 1). Two major immunoreactive bands of 57 ± 1 kDa and 31 ± 1 kDa were detected (Fig. 1). Two minor bands at 53 and 50 kDa and various bands smaller than 21 kDa were also identified (Fig. 1). Similar results were obtained with HEK-293 cells expressing the rat H_2Flag receptor (data not shown). The specificity of the anti-Flag M2 monoclonal antibody was confirmed by the measurement of cellular immunofluorescence. Neither pRK_5- nor pRK_5humH_2 transfected HEK-293 cells showed considerable immunoreactivity. In contrast, HEK-293 cells transiently expressing the human or rat H_2Flag receptor showed a clear immunofluorescence signal in the plasma membrane (data not shown). Permeabilization of the transiently expressed 293 cells with the human as well as rat H_2Flag receptor also showed immunofluorescence in the cytoplasm in areas corresponding to the Golgi apparatus (cover thesis).

Stable expression of the rat histamine H_2Flag receptor in HEK-293 cells

In view of our interest in the regulation of the rat H_2 receptor expression (Chapter 5 and 6), the rat H_2Flag receptor was stably expressed in HEK-293 cells. Cotransfection of HEK-293 cells with the plasmids pRK_5ratH_2Flag and pSV_2neo by lipofection resulted after 10 to 14 days of selection in culture medium, supplemented with 500 µg/ml G418, in the formation of several clonal cell lines. A clone ($293rH_2Flag$) that expressed 1594 ± 17 fmol/mg protein [^{125}I]-APT binding sites (mean ± s.e.mean, n = 3) was selected. Initial experiments indicated again that the

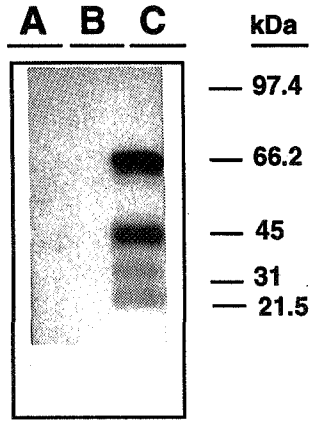

Fig. 1. Western blot of human H_2Flag receptors in pRK_5- (lane A), pRK_5H_2 (lane B) and pRK_5H_2Flag (lane C) transfected HEK-293 cells. Solubilized membrane proteins were subjected to 10 % SDS/PAGE electrophoresis and immunoblotted using the mouse monoclonal anti-Flag M2 antibody. Molecular weight markers are indicated. Similar results were obtained in two other experiments.

Flag-epitope did not alter the pharmacological characteristics of the H_2 receptor protein (M. J. Smit, unpublished observations). In the 293rH_2Flag cells the H_2Flag receptor was functionally coupled to adenylate cyclase. A 10 mins incubation with histamine resulted in a maximal increase of the intracellular cAMP levels from 51 ± 12 pmol/well to 805 ± 202 pmol/well (mean ± s.e.mean, n = 6), with a pD_2 value of 7.3 ± 0.06 (mean ± s.e.mean, n = 3). Prolonged incubation (24 hrs) of the 293rH_2Flag cells with 1 or 100 μM histamine resulted in a dose-dependent reduction of the H_2 receptor expression (Fig. 2). Stimulation of 293rH_2Flag cells with 10 μM forskolin also resulted in a marked increase of cellular cAMP (991 ± 293 pmol/well), but only a moderate reduction of the H_2 receptor expression was observed after a 24 hrs incubation with 10 μM forskolin (Fig. 2).

Using the anti-Flag M2 monoclonal antibody the H_2Flag receptor could be visualized in the membranes of 293rH_2Flag cells (Fig. 3 A, B). Permeabilization of the 293rH_2Flag cells also showed immunofluorescence in the cytoplasm (data not shown). One hr pretreatment of the 293rH_2Flag cells with 100 μM histamine at 37 °C resulted in an almost complete disappearance of the immunoreactivity in the cell membrane (Fig. 3 C). Yet, at this time point no effective receptor downregulation was observed. After 1 hr treatment with 100 μM histamine the [^{125}I]-APT binding remained unchanged (103 ± 7 % compared to non-treated 293rH_2Flag cells, mean ± s.e.mean, n = 4). Immunofluorescence of epitope-tagged H_2 receptors was already detected inside the cell and it was impossible to identify a further increase of the intracellular immunofluorescence.

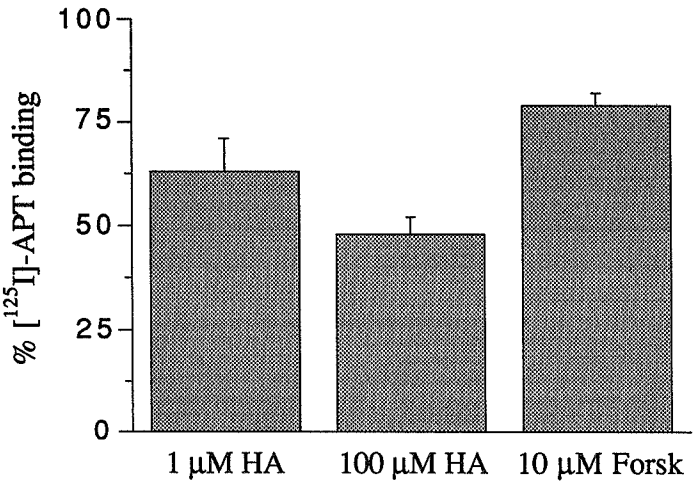

Fig. 2. Downregulation of the rat H_2Flag receptor stably expressed in 293rH_2Flag cells. Cells were incubated with the compounds for 24 hrs and analysed for [^{125}I]-APT binding. The binding was expressed as the percentage of non-treated 293rH_2Flag cells. Data shown are mean ± s.e.mean of six independent experiments.

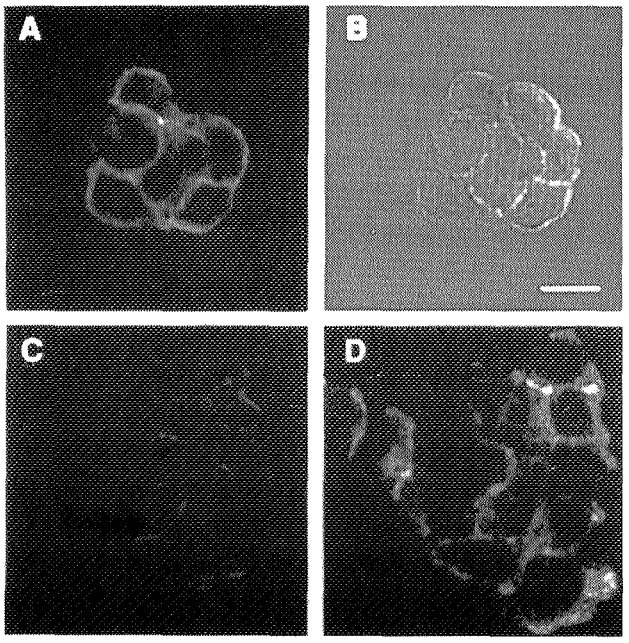

Fig. 3. Internalization of the rat H_2Flag receptor in 293rH_2Flag cells as visualized by immunofluorescence. Panel A and B show the immunofluorescence and light microscopic image of the same group fixed control 293rH_2Flag cells respectively. Panel C and D show the immunofluorescence after treatment of 293rH_2Flag cells with 100 μM histamine at 37 °C for 1 hr and the same treatment with histamine after 10 mins preincubation with 80 μM phenylarsine oxide respectively. Data shown are a representative example of three independent experiments. The bar represents 15 μm.

The rapid histamine-induced disappearance of the Flag-immunoreactivity was effectively blocked by a 10 mins pretreatment of 293rH_2Flag cells with 80 μM phenylarsine oxide (Fig. 3 D). In a separate set of experiments the reversibility of the observed disappearance of Flag-immunoreactivity was investigated. After a one hr incubation of 293H_2Flag cells with 100 μM histamine at 37 °C the cells were extensively washed with DMEM/50 mM HEPES (pH = 7.4). Thereafter the cells were allowed to recover for two hrs at 37 °C in the same medium. This procedure allowed the detection of the recovery of Flag-immunoreactivity in the cell membranes (compare Fig. 4 A, C). Finally, we investigated the effect of forskolin pretreatment on H_2Flag receptor internalization. A one hr incubation of 293H_2Flag cells with 10 μM forskolin at 37 °C did not lead to important changes of the membrane-localized Flag immunoreactivity (compare Fig. 4 A, B and D).

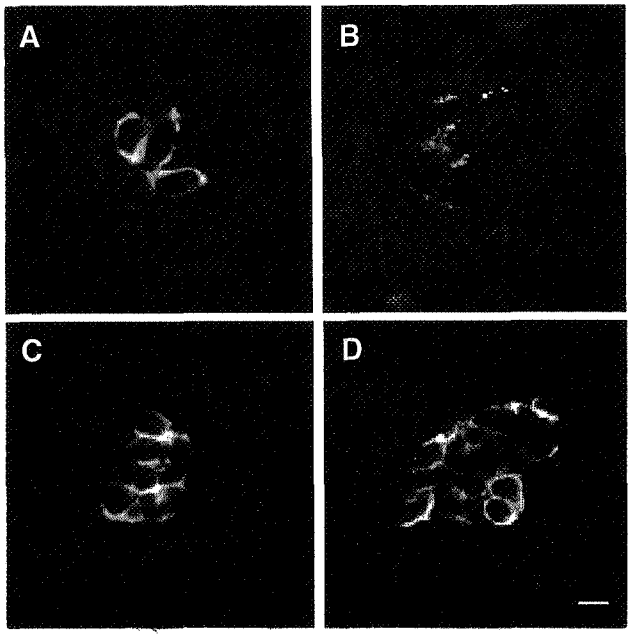

Fig. 4. Internalization of the rat H_2Flag receptor in 293rH_2Flag cells as visualized by immunofluorescence. Panel A shows the immunofluorescence of fixed control 293rH_2Flag cells, whereas in panel B the immunofluorescence after treatment of 293rH_2Flag cells with 100 μM histamine at 37 °C for 1 hr is shown. In panel C the cells were treated as in B but extensively washed and incubated for additional two hrs at 37 °C. In panel D 293rH_2Flag cells were treated with 10 μM forskolin at 37 °C for 1 hr. Data shown are a representative example of three independent experiments. The bar represents 15 μm.

Discussion

In the present study we epitope-tagged the H_2 receptor to detect the receptor protein by Western blotting and immunohistochemistry. As shown by [^{125}I]-APT binding studies and cAMP measurements the introduction of the Flag peptide at the N-terminus did not modify the pharmacological properties of the H_2 receptor, validating our experimental approach. The Flag epitope allowed the immunological detection of the H_2 receptor protein. Using HEK-293 cells expressing the wild-type receptor no immunoreactive bands were observed on a Western blot, whereas with cells expressing the H_2Flag receptor two main bands of 57 and 31 kDa peptides were found to be labelled. Some minor bands at 53-50 kDa were also found and probably represent partially glycosylated receptors, whereas the 31 kDa peptide is likely the result of a

proteolytic breakdown of the 57 kDa peptide, since omittance of protease inhibitors largely increased the intensity of this band (data not shown). In literature some conflicting results have been reported on the molecular identification of the H_2 receptor proteins (Fukushima *et al.*, 1994, Ruat *et al.*, 1990). Using [^{125}I]-iodoazidopotentidine Ruat *et al.* (1990) reported the photoaffinity labelling of 87, 59, 51 and 32 kDa proteins in guinea-pig brain membranes. Only the labelling of the 59 and 32 kDa bands was prevented by coincubation with H_2 antagonists (Ruat *et al.*, 1990). In another study an anti-peptide antibody directed against the C-terminus of the canine H_2 receptor was used to probe Western blots of CHO cells transfected with the canine H_2 receptor (Fukushima *et al.*, 1994). The antibody recognized a broad band with an apparent molecular mass of 63-95 kDa (Fukushima *et al.*, 1994), which is substantially higher than the molecular mass found in the present study. Yet, the results obtained after photoaffinity labelling (Ruat *et al.*, 1990) correspond well with our results with the anti-Flag antibody. The reasons for the discrepancy with the results obtained with the anti-peptide antibody (Fukushima *et al.*, 1994) are not clear, but are probably due to technical differences in the solubilization step. In the present study we used the same protocol as Ruat *et al.* (1990), but in preliminary experiments we observed that the migration of Flag-immunoreactive bands was dependent on the solubilization protocol used (M. J. Smit, unpublished observations).

The introduction of the Flag peptide allowed us to demonstrate H_2 receptor internalization for the first time. A short incubation of stably transfected HEK-293 cells with histamine at 37°C resulted in the rapid disappearance of Flag immunoreactivity in the plasma membrane. Apparently, agonist stimulation of the H_2 receptor results in the rapid translocation of the H_2 receptor to intracellular domains, which are inaccessible for the anti-Flag antibody. Our data indicate that under these conditions these receptor proteins are not degraded, but recycled. The internalization was rapidly reversed by washing and incubation at 37 °C, whereas [^{125}I]-APT binding studies did not reveal major changes in the total number of H_2 receptors after a one hr treatment with histamine. The loss of Flag-immunoreactivity was moreover effectively inhibited by preincubation with the endocytosis inhibitor phenylarsine oxide. This compound is reported to inhibit endocytosis of e.g. recycling epidermal growth factor receptors (Hertel *et al.*, 1985), but more interesting also inhibits the internalization of the β_2-adrenergic and substance P NK_1 receptors (Feldman *et al.*, 1986, Garland *et al.*, 1994, Hertel *et al.*, 1985). Treatment of the 293rH_2 Flag cells with forskolin did not result in H_2 receptor internalization, although production of cAMP and H_2 receptor downregulation was observed. These data indicate that the process of H_2 receptor internalization in HEK-293 cells is cAMP-independent, as previously has been found for the β_2-receptor (Bouvier *et al.*, 1989). Moreover, the different extents of H_2 receptor downregulation by forskolin and histamine also indicates a cAMP-independent mechanism of H_2 receptor downregulation in these cells. Recently, we observed similar mechanisms in CHO cells, transfected with mutant H_2 receptors (Chapter 5), substantiating the hypothesis of cAMP-independent pathways of H_2 receptor regulation.

The exact fate of the internalized H_2 receptors is not clear, but internalization via clathrin-

coated pits and recycling to the plasma membrane of receptors of e.g transferrin and the epidermal growth factor has been shown (Hertel *et al.*, 1985, Von Zastrow and Kobilka, 1992). Recently, Von Zastrow and Kobilka provided evidence that the β_2-receptor is internalized and recycled via such a pathway (Von Zastrow and Kobilka, 1992). The functional role of GPCR internalization is not completely resolved, but there is strong evidence that internalization of the β_2-receptor is required for receptor resensitization. Sibley *et al.* (1986) reported that high phosphatase levels were associated with sequestered vesicles. Consequently, the sequestered β_2-receptors were phosphorylated to a lesser extent than desensitized β_2-receptors (Sibley *et al.*, 1986). Moreover, inhibition of GPCR internalization resulted in an inhibition of receptor resensitization (Barak *et al.*, 1994, Palmer *et al.*, 1994, Pippig *et al.*, 1995, Yu *et al.*, 1993), corroborating the suggestion that internalization is required for receptor dephosphorylation and resensitization. Homologous desensitization of the H_2 receptor responsiveness has been shown previously (Arima *et al.*, 1993, Fukushima *et al.*, 1994, Schreurs *et al.*, 1984, Smit *et al.*, 1994), but H_2 receptor phosphorylation and resensitization has so far not been studied. Our approach of epitope-tagging of the H_2 receptor should allow to investigate this process. This approach should also enable to investigate which structural elements of the receptor protein are implicated in H_2 receptor internalization. Serine/threonine-rich domains in the third intracellular loop or C-terminus and a highly conserved NP(X)Y motif in the seventh transmembrane domain have been shown to be involved in the internalization of GPCRs (Barak *et al.*, 1994, Campbell *et al.*, 1991, Hunyady *et al.*, 1994, Lameh *et al.*, 1992). Similar structural features are found in the rat H_2 receptor amino acid sequence (Ruat *et al.*, 1991), but the assignment of their role awaits site-directed mutagenesis studies.

In conclusion, receptor internalization is a prominent mechanism of H_2 receptor regulation. This process is rapid, reversible, cAMP-independent and is probably responsible for the resensitization of desensitized H_2 receptors.

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Chapter 9

Inverse agonism of histamine H_2 antagonists leads to antagonist-induced upregulation of the histamine H_2 receptor

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In the present study we have demonstrated that the wild-type rat histamine H_2 receptor density in Chinese Hamster Ovary cells ($CHOrH_2WT$ cells) is affected by long-term exposure to some H_2 antagonists. Prolonged treatment of $CHOrH_2WT$ cells with cimetidine resulted in time-dependent ($t_{1/2}$ = 3.5 hrs, concentration of 100 μM cimetidine) and dose-dependent (EC_{50} = 6.0 μM at 24 hrs incubation) increase of [^{125}I]-iodoaminopotentidine ([^{125}I]-APT) binding, referred to as H_2 receptor upregulation (68 ± 2% maximum upregulation). Yet, the cimetidine analogue VUF 8299, which is devoid of H_2 receptor antagonistic activity on the guinea-pig right atrium, induced no H_2 receptor upregulation. No change in H_2 receptor density was observed either after long-term treatment of $CHOrH_2WT$ cells with the H_1 antagonist triprolidine or the H_3 antagonist thioperamide.

Basal levels of cAMP and forskolin-induced formation of cAMP were shown to increase upon elevation of H_2 receptor density (286 fmol/mg protein and higher), indicating that the wild-type H_2 receptor displays properties resembling those of constitutively active GPCR mutants. Interestingly, the agonist-independent H_2 receptor activity in $CHOrH_2WT$ was accompanied by the occurrence of inverse agonism exerted by cimetidine, whereas its inactive structural analogue VUF 8299 did not show negative intrinsic activity. Cimetidine did not display inverse agonism in $CHOrH_2Leu^{124}Ala$ (CHO cells expressing the mutant $H_2Leu^{124}Ala$ receptor which was previously found to be uncoupled from its G_s-protein (Chapter 5)) and $CHOrH_2WT6$ cells (density 96 ± 26 fmol/mg protein), both showing reduced basal and forskolin-induced levels of cAMP compared to $CHOrH_2WT$ cells. Accordingly, cimetidine only induced H_2 receptor upregulation in $CHOrH_2WT$ (density 975 ± 12 fmol/mg protein) and $CHOrH_2WT9$ (density 286 ± 52 fmol/mg protein) cells and not in $CHOrH_2Leu^{124}Ala$ or $CHOrH_2WT6$ cells. Moreover, not all H_2 antagonists displayed negative intrinsic activity. Burimamide, for example, acted as a neutral antagonist as no major changes in basal cAMP levels or in H_2 receptor density were observed after incubation with burimamide.

Based on these findings we conclude that at physiological H_2 receptor densities H_2 receptors are precoupled and that several H_2 antagonists, previously thought to act as competitive antagonists, may actually function as inverse agonists. The displayed inverse agonism of some H_2 antagonists appears to be the mechanistic basis for the H_2 antagonist-induced H_2 receptor upregulation in $CHOrH_2WT$ cells. Moreover, it may also explain the previously reported observations after long-term H_2 antagonist treatment of gastric ulcers, such as increased sensitization of the H_2 receptor, increased intragastric hyperacidicity and loss of antisecretory effect. Our data imply a careful reconsideration of the pharmacological activity of the clinically used H_2 antagonists. In some instances neutral antagonists may be favoured.

Introduction

G-protein coupled receptors (GPCRs) are known to undergo receptor down- or upregulation after excessive stimulation or prolonged blockade respectively. These processes have been observed *in vivo* (Elfellah and Reid, 1989, Homcy *et al.*, 1991, Motomura *et al.*, 1990, Reid *et al.*, 1994, Yoburn *et al.*, 1994) and have been suggested to have consequences for drug therapy (Brodde *et al.*, 1990, Ghosh *et al.*, 1991, Homcy *et al.*, 1991, Motomura *et*

al., 1990, Stiles, 1991).

In Chapter 5 we describe that histamine H_2 receptors are affected by long-term agonist-exposure, as shown by H_2 receptor downregulation in transfected Chinese Hamster Ovary (CHO) cells. Moreover, Diaz *et al.* (1994) suggested that *in vivo* receptor downregulation might explain the inverse relationship between H_2 receptor expression and the localization of histamine-synthesizing cells in the rodent gastric wall.

Clinically, histamine H_2 antagonists are of great importance. These drugs (cimetidine, ranitidine, famotidine, nizatidine) are widely used in the treatment of gastric ulcers (Bertaccini and Coruzzi, 1992), whereas the reported beneficial effect of famotidine in schizophrenia (Deutsch *et al.*, 1993, Kaminsky *et al.*, 1990) might further boost their therapeutic use. Interestingly, *in vivo* modulation of H_2 receptor responsiveness and H_2 receptor density after treatment of H_2 antagonists has also been reported (Bertaccini and Coruzzi, 1992, Coruzzi and Bertaccini, 1989, Martinez-Mir *et al.*, 1993, Merki and Wilder-Smith, 1994, Nwokolo *et al.*, 1991). Chronic treatment of conscious cats with ranitidine resulted in increased parietal cell sensitivity (Coruzzi and Bertaccini, 1989). Furthermore, abrupt withdrawal of histamine H_2 receptor blockade (ranitidine, cimetidine, nizatidine) in humans was shown to lead to intragastric hyperacidity (Nwokolo *et al.*, 1991). Others reported a loss of antisecretory effect after prolonged infusions of ranitidine (Merki and Wilder-Smith, 1994). As for alterations in H_2 receptor expression, it has been reported that the histamine H_2 receptor expression in brain tissue (globus pallidus) of schizophrenic patients which receive chronic neuroleptic treatment is increased (Martinez-Mir *et al.*, 1993). Some neuroleptics show considerable affinity for human H_2 receptors (Leurs *et al.*, 1994, Traiffort *et al.*, 1992), indicating the possible *in vivo* regulation of H_2 receptor expression upon treatment with neuroleptics.

Despite the therapeutical importance of H_2 antagonists so far no detailed insights on the modulation of H_2 receptor function by H_2 antagonists have been obtained. According to classical models for drug-GPCR interaction, competitive antagonists are believed to simply inhibit the binding of agonists to the receptors (De Lean *et al.*, 1980). Their physiological effect, including receptor upregulation, is therefore attributed to their ability to prevent activation/downregulation of receptors by endogenous hormones or neurotransmitters. However, there is now substantial evidence that contradicts this notion (Lefkowitz *et al.*, 1993, Schütz and Freissmuth, 1992). For some GPCRs some antagonists were shown to induce effects opposite to those observed by agonists, thereby displaying negative intrinsic activity, also referred to as inverse agonism (Lefkowitz *et al.*, 1993, Schütz and Freissmuth, 1992). Consequently, unoccupied receptors were proposed to spontaneously isomerize between 'inactive' and 'activated' states (Costa *et al.*, 1992, Lefkowitz *et al.*, 1993, Onaran *et al.*, 1993). Agonists were shown to favour the active state of the receptor (coupled to the G-protein), whereas inverse agonists would favour the inactive state of the receptor. Initial conclusive evidence of inverse agonism by GPCR antagonists came from experiments with constitutively active GPCR mutants (α_{1B}-, α_2-C10- and β_2-adrenergic receptors) (Cotecchia

et al., 1990, Kjelsberg *et al.*, 1992, Ren *et al.*, 1993, Samama *et al.*, 1994). These GPCR mutants were shown to activate signal transduction pathways without agonist stimulation (Cotecchia *et al.*, 1990, Kjelsberg *et al.*, 1992, Ren *et al.*, 1993, Samama *et al.*, 1994). Intriguingly, this spontaneous activity could be reduced by receptor antagonists. Thereafter, wild-type serotonine 5-HT_{2C} (Barker *et al.*, 1994), α_{2D}-adrenergic (Tian *et al.*, 1994), β_2-adrenergic (Chidiac *et al.*, 1994), dopamine D_{1B} (Tiberi and Caron, 1994) and bradykinin B_2 receptors (Leeb-Lundberg *et al.*, 1994) were shown to display agonist-independent activity as well. These observations suggest that the agonist-independent activity may be a natural feature of some GPCRs. Consequently, a dramatic change in our views on the pharmacological activity of some GPCR antagonists will be needed.

In the present study we examined the effects of H_2 antagonists on rat histamine H_2 receptor activity and H_2 receptor expression in transfected CHO cells (Traiffort *et al.*, 1992). Using CHO cell lines expressing different levels of H_2 receptor proteins and a CHO cell line expressing a mutant H_2 receptor, which was previously (Chapter 5) shown to be uncoupled of its G_s-protein, we have evidence that several H_2 antagonists act as inverse agonists and consequently cause H_2 receptor upregulation. These observations might have important therapeutical consequences for the currently available H_2 antagonists and the future development of effective drug therapy.

Materials and methods

Cell culture and transfection CHO cells, deficient in dihydrofolate reductase, were maintained in a humidified atmosphere with 5% CO_2 in Dulbeco's Modified Eagle's Medium (DMEM), containing 10% (vol/vol) foetal calf serum, supplemented with 2 mM L-glutamine, MEM amino acids, 50 IU/ml penicillin, 50 µg/ml streptomycin and hypoxanthine. CHO cells were stably transfected with 15 µg of the eukaryotic expression vector pSVrH_2 using Transfectam (Promega). After two weeks of selection in medium without hypoxanthin and thymidine and with 10 % dialyzed foetal calf serum, surviving CHO colonies were isolated by ring cloning. Two clones expressing 96 ± 26 and 286 ± 52 fmol H_2 receptors/mg protein, referred to as CHOrH_2WT6 and CHOrH_2WT9 respectively, were selected and grown at 37°C in a humified atmosphere with 5% CO_2 in DMEM, containing 10% (vol/vol) dialyzed foetal calf serum, supplemented with 2 mM L-glutamine, MEM amino acids, 50 IU/ml penicillin and 50 µg/ml streptomycin. The same medium was used for CHO cells expressing the wild-type rat histamine H_2 receptor (CHOrH_2WT) (975 ± 12 fmol/mg protein) (Traiffort *et al.*, 1992) and rat histamine H_2Leu^{124}Ala (CHOrH_2Leu^{124}Ala) (980 ± 7 fmol/mg protein) (Chapter 5).

Biochemical measurements The determination of H_2 receptor binding and levels cAMP were performed as described in Chapter 5. Instead of seeding the cells in 24-well plates, CHOrH_2WT, CHOrH_2WT6, CHOrH_2WT9 and CHOrH_2Leu cells were seeded in 12-well plates for the measurement of basal cAMP production.

RNA slot blot analysis RNA was analyzed as described in Chapter 5.

Chemicals Histamine dihydrochloride, isobutylmethylxanthine (IBMX), cyclic AMP (cAMP), forskolin and triprolidine dihydrochloride was obtained from Amersham. Aminopotentidine, VUF 8299 and thioperamide maleate were taken from laboratory stock. Gifts of cimetidine and burimamide (SmithKline Beecham, United Kingdom), tiotidine (Imperial Chemical Industries, United Kingdom), ranitidine dihydrochloride (Glaxo, United Kingdom) and famotidine (Merck Sharp & Dohme, The Netherlands) are greatly acknowledged.

Statistical analysis All data shown are expressed as mean ± standard error (mean ± s.e.mean) of at least three independent experiments. Statistical analysis was carried out by Student's *t*-test. P-values < 0.05 were considered to indicate a significant difference.

Results

Effect of long-term incubation of H_2 antagonists on [^{125}I]-APT binding in CHOrH$_2$WT cells

As we showed in previously (Chapter 5) exposure of CHOrH$_2$WT cells to 100 μM of histamine for prolonged periods of time resulted in a time-dependent decrease of [^{125}I]-iodoaminopotentidine ([^{125}I]-APT) binding sites (44 ± 10%, mean ± s.e.mean, n = 4, $p < 0.05$) (Fig. 1). The histamine-induced downregulation (24 hrs incubation with 100 μM of histamine) was blocked by co-incubation of CHOrH$_2$WT cells with 10 μM of the H_2 antagonist tiotidine, as no change in [^{125}I]-APT binding sites was observed (Inset Fig. 1). Remarkably, exposure of CHOrH$_2$WT cells to tiotidine (10 μM) alone surprisingly resulted in a significant increase in [^{125}I]-APT binding (82 ± 6%, n = 4, mean ± s.e.mean, $p < 0.05$, Inset Fig. 1, Table 1). Pretreatment of CHOrH$_2$WT cells for 24 hrs with other H_2 antagonists, such as cimetidine, ranitidine and aminopotentidine, possessing distinct structural properties led to similar observations (Table 1). Pretreatment of CHOrH$_2$WT cells with different H_2 receptor antagonists resulted in a significant increase in [^{125}I]-APT binding (Table 1). The observed increase of [^{125}I]-APT binding was ascribed to an increase in the maximal number of [^{125}I]-APT binding sites when CHOrH$_2$WT cells were incubated with 10 μM aminopotentidine for 24 hrs (Table 2).

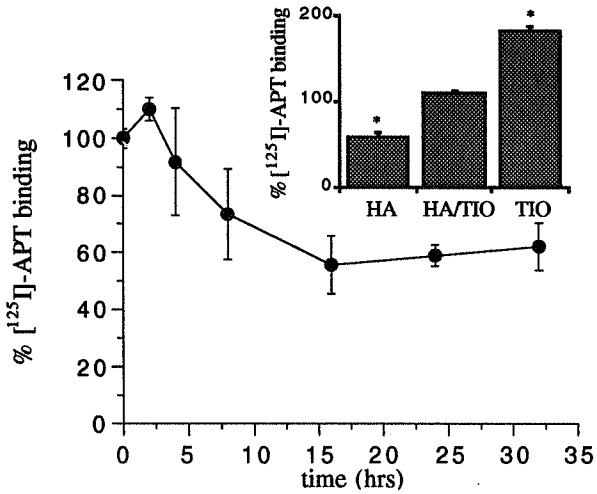


Fig. 1. Histamine-induced decrease of [^{125}I]-APT binding in CHOrH$_2$WT cells. CHOrH$_2$WT cells were incubated with 100 μM histamine for the indicated times and [^{125}I]-APT binding in membranes was measured. The [^{125}I]-APT binding is expressed as a percentage of [^{125}I]-APT binding measured in non-treated histamine. The data shown represent the means ± s.e.mean of 4 independent experiments. Inset. Effect of the H_2 antagonist tiotidine on the histamine-induced H_2 receptor downregulation and [^{125}I]-APT binding. CHOrH$_2$WT cells were incubated in the presence of 100 μM histamine (HA), 10 μM tiotidine (TIO) together with 100 μM histamine and 10 μM tiotidine alone for 24 hrs. Data shown are means ± s.e.mean of 4 independent experiments. Significant differences from control, represented by the number of [^{125}I]-APT binding sites measured in non-treated cells, at $p < 0.05$ (Student's *t*-test) are indicated by the asterisks.

Table 1 Effect of 24 hrs incubation of H_1, H_2 and H_3 antagonists on [^{125}I]-APT binding in CHOrH_2WT cells

Antagonist		% upregulation
Tiotidine	(10 μM)	82 ± 6 *
Cimetidine	(100 μM)	68 ± 9 *
Aminopotentidine	(10 μM)	79 ± 9 *
Triprolidine	(1 μM)	-3 ± 10
Thioperamide	(1 μM)	12 ± 11

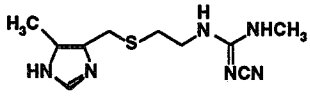
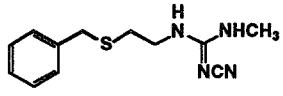
CHOrH_2WT were incubated for 24 hrs with the indicated drugs and [^{125}I]-APT binding in membranes was measured. The [^{125}I]-APT binding is expressed as a percentage of upregulation compared to non-treated cells. Data were calculated as means ± s.e.mean from at least 3 experiments. The asterisk indicates a significant difference ($p < 0.05$) from control, represented by non-treated cells.

Table 2 Characteristics of [^{125}I]-APT binding to rat histamine H_2 receptors expressed in CHO cells pretreated for 24 hrs with or without aminopotentidine (APT)

pretreatment 24 hrs	K_d (nM)	B_{max} (fmol/mg protein)
control	0.43 ± 0.06	975 ± 12
APT (10 μM)	0.54 ± 0.02	1768 ± 85 *

The dissociation constant (K_d) and maximal number of binding sites (B_{max}) was determined by using a non-linear fitting according to a one-site binding mode. The data shown represent the means ± s.e.mean of 3 independent experiments. The asterisk indicates a significant difference ($p < 0.05$) from control, represented by non-treated cells.

Table 3 Effect of 24 hrs incubation of CHOrH_2WT cells with respectively 100 μM cimetidine and 100 μM of the inactive analogue of cimetidine VUF 8299 on [^{125}I]-APT binding to CHOrH_2WT membranes

		pA_2 (atrium)	% upregulation
cimetidine		6.2 ± 0.1	85 ± 9*
VUF 8299		< 4	-3 ± 27

The antagonistic activity, depicted by a pA_2 value, of cimetidine and VUF 8299 was determined on the guinea-pig atrium (Sterk *et al.*, 1987). CHOrH_2WT were incubated for 24 hrs with 100 μM cimetidine and 100 μM of VUF 8299 and [^{125}I]-APT binding in membranes was measured. The [^{125}I]-APT binding is expressed as a percentage of upregulation compared to non-treated cells. The values are means ± s.e.mean of 3 independent determinations. The asterisk indicates a significant difference ($p < 0.05$) from control, represented by non-treated cells.

Pretreatment of CHOrH$_2$WT cells for 24 hrs with 100 μM VUF 8299, a structural analogue of cimetidine devoid of H_2 antagonistic activity at the guinea-pig right atrium (Sterk *et al.*, 1987), did not affect the [^{125}I]-APT binding (Table 3), suggesting that the observed effects of cimetidine were related to its H_2 antagonistic properties. Moreover, cimetidine had no effect on the density of guinea-pig H_1 receptors (gpH$_1$) expressed in CHO cells (untreated cells: 100 ± 2% gpH$_1$ receptors, cimetidine-treated cells: 91 ± 4% gpH$_1$ receptors, mean ± s.e.mean, n = 4) using the same pSV expression vector. In addition, the histamine H_1 and H_3 antagonists, triprolidine (1 μM) and thioperamide (1 μM) did not affect the amount of H_2 receptors of CHOrH$_2$WT cells after 24 hrs incubation (Table 1).

Incubation of CHOrH$_2$WT cells for 24 hrs with increasing concentrations of cimetidine led to a dose-dependent increase of [^{125}I]-APT binding (Fig. 2A). The EC_{50} was found to be 6.0 ± 1.1 μM (mean ± s.e.mean, n = 4). Exposure of CHOrH$_2$WT cells to 100 μM of cimetidine for prolonged periods of time resulted in a time-dependent increase of [^{125}I]-APT binding (Fig. 2B). A maximal increase of [^{125}I]-APT binding of 68 ± 2% (mean ± s.e.mean, n = 3) compared to non-treated cells (mean ± s.e.mean, n = 3, $p < 0.05$) was observed after 12 hrs incubation of cells with 100 μM cimetidine. Half maximal increase of [^{125}I]-APT binding was recorded at an incubation period of approximately 3.5 hrs.

H_2 receptor mRNA levels in cimetidine-treated CHOrH$_2$WT cells

Exposure of CHOrH$_2$WT cells to 100 μM of cimetidine for increasing periods of time resulted in a rapid significant transient increase of H_2 receptor mRNA (maximal increase of 32 ± 8%,

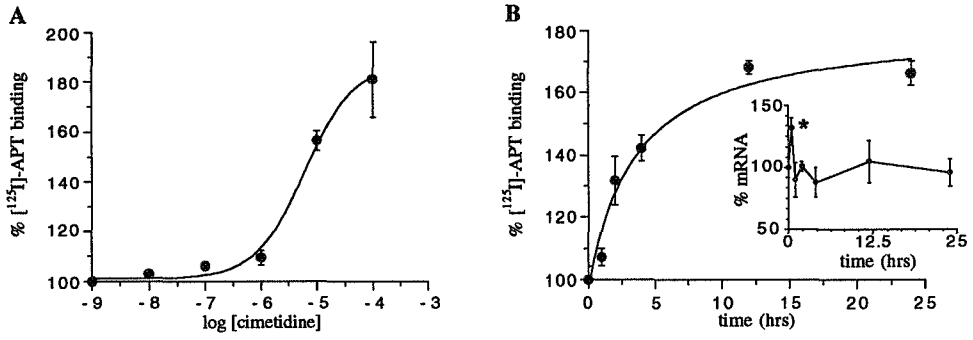


Fig. 2. A, Dose-dependent increase of [^{125}I]-APT binding induced by cimetidine in CHOrH$_2$WT cells. CHOrH$_2$WT cells were exposed to various concentrations of cimetidine for 24 hrs. The data represent the mean ± s.e.mean of 4 independent experiments. B, Time-dependent increase of [^{125}I]-APT binding in CHOrH$_2$WT cells by 100 μM cimetidine. CHOrH$_2$WT cells were incubated with 100 μM cimetidine for the indicated times and [^{125}I]-APT binding in membranes was measured. The [^{125}I]-APT binding is expressed as a percentage of [^{125}I]-APT binding measured in non-treated histamine. The data shown represent the mean ± s.e.mean of 3 independent experiments. Inset. Cimetidine-induced modulation of H_2 receptor mRNA levels in CHOrH$_2$WT cells. CHOrH$_2$WT cells were incubated for the indicated times with 100 μM of cimetidine. Cells were harvested, total RNA was extracted and quantified by means of a RNA slot blot assay as described in Chapter 5. The results displayed are the mean ± s.e.mean of three separate experiments, performed in duplicate.

mean ± s.e.mean, n = 6, $p < 0.05$) (Inset Fig. 2B). This effect was at its peak after 0.5 hrs of incubation of cells with cimetidine (100 μM). Thereafter, the amount of H_2 receptor mRNA returned to control mRNA levels.

Basal cAMP levels and forskolin-induced formation of cAMP in untransfected CHO cells and CHO cell lines expressing H_2 receptors

In order to examine whether the H_2 antagonist-induced upregulation of [^{125}I]-APT binding sites can be ascribed to an inhibition of basal H_2 receptor activity we determined the basal levels and forskolin-induced formation of cAMP in CHO cells expressing different levels of rat histamine H_2 receptors. As can be seen in Table 4 the basal level of cAMP in untransfected CHO cells is the lowest and rises upon an increase of expressed H_2 receptors. The forskolin (10 μM)-induced formation of cAMP was also found to increase upon elevation of the H_2 receptor density (Table 4). In contrast, basal levels and forskolin-induced formation of cAMP in CHO cells expressing a mutant H_2 receptor, which was previously found to be uncoupled from its G_s protein (H_2Leu^{124}Ala, see Chapter 5), were found to be significantly lower than those recorded in CHOrH_2WT cells, expressing the same amount of H_2 receptors (Table 4).

Table 4 Basal cAMP levels and forskolin-induced formation of cAMP in untransfected cell lines and cell lines expressing H_2 receptors and effect of long-term incubation of these cells with cimetidine on [^{125}I]-APT binding

cell line	H_2 receptor density (fmol/mg protein)	cAMP (pmol/mg protein)		% upregulation
		basal	10 μM forskolin	
CHO	0	37 ± 4	2148 ± 169	
CHOrH_2WT6	96 ± 26	42 ± 7	2054 ± 393	0 ± 3
CHOrH_2WT9	286 ± 52	62 ± 5 *	2472 ± 130 *	83 ± 9 *
CHOrH_2WT	975 ± 12	198 ± 17 *	2945 ± 132 *	68 ± 9 *
CHOrH_2Leu^{124}Ala	980 ± 7	44 ± 3 **	2123 ± 71 **	18 ± 8 **

The basal cAMP levels and forskolin (10 μM)-induced formation of cAMP in different CHO cell lines was determined for 10 mins at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes, pH 7.4. Data represent the mean ± s.e.mean of at least 3 independent experiments performed in duplicate. The asterisk and the double asterisk indicate a significant difference ($p < 0.05$) from untransfected CHO cells and CHOrH_2WT cells respectively. Column %upregulation; the different CHO cell lines were incubated for 24 hrs with 100 μM cimetidine and [^{125}I]-APT binding in membranes was measured. The [^{125}I]-APT binding is expressed as a percentage of upregulation compared to non-treated cells. Data were calculated as means ± s.e.mean from at least 3 experiments. The asterisk and double asterisk indicate a significant difference ($p < 0.05$) from non-treated cells and CHOrH_2WT cells respectively.

Effect of cimetidine on basal cAMP levels in various CHO cell lines

In view of the observed increase of basal activity in CHOrH_2WT6 and CHOrH_2WT9 and

CHOrH_2WT cells compared to untransfected CHO cells we were interested to see whether cimetidine was able to exhibit negative intrinsic activity by reducing the basal levels of cAMP. Incubation of CHOrH_2WT cells with increasing concentrations of cimetidine resulted in a dose-dependent decrease of levels of cAMP (Fig. 3A). The EC_{50} value of this effect was 1.2 ± 0.3 μM (mean ± s.e.mean, n = 4). Cimetidine decreased the basal activity in CHOrH_2WT cells for 72 ± 6%. The inactive cimetidine VUF 8299, on the other hand, exhibited negative intrinsic activity at a concentration of 100 μM only (Fig. 3A).

Basal levels of cAMP (62 ± 5 pmol cAMP/mg of protein, mean ± s.e.mean, n = 5) in CHOrH_2WT9 cells were also found to be significantly reduced by 100 μM cimetidine (48 ± 5 pmol/mg of protein, 23% reduction, mean ± s.e.mean, n = 3), while no effect was observed when CHOrH_2WT6 cells were exposed to 100 μM cimetidine (basal cAMP level: 42 ± 7 pmol/mg of protein, level of cAMP after cimetidine treatment: 46 ± 9 pmol/mg of protein, mean ± s.e.mean, n = 4). In CHOrH_2Leu^{124}Ala cells, cimetidine did not exhibit any negative intrisic activity either (Fig. 3B). Moreover, no effect on basal cAMP levels was observed when untransfected CHO cells were exposed to 100 μM cimetidine (basal cAMP level: 37 ± 4 pmol/mg of protein, level of cAMP after cimetidine treatment: 39 ± 2 pmol/mg of protein, mean ± s.e.mean, n = 5)

Effect of cimetidine on [^{125}I]-APT binding in various CHO cell lines, expressing H_2 receptors

As observed earlier (Fig. 2A, B, Table 1, 3), 24 hrs incubation of CHOrH_2WT cells with 100 μM cimetidine resulted in a marked increase of [^{125}I]-APT binding sites. Similarly, 24 hrs incubation of CHOrH_2WT9 cells with 100 μM cimetidine led to a significant increase in

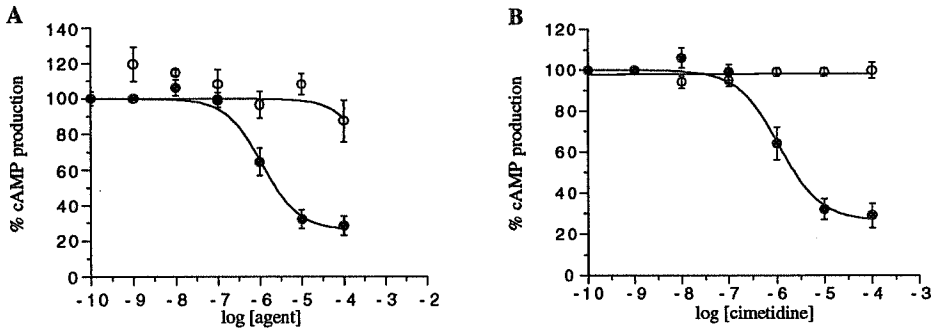


Fig. 3. A, Effect of cimetidine and inactive analogue VUF 8299 on basal levels of cAMP in CHOrH_2WT cells. CHOrH_2WT cells were exposed to increasing concentrations of cimetidine (filled circles) or VUF 8299 (open circles) for 10 mins at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes, pH 7.4. Data represent the mean ± s.e.mean of 4 independent experiments. B, Effect of cimetidine on basal levels of cAMP in CHOrH_2WT cells and CHOrH_2Leu^{124}Ala cells. CHOrH_2WT (filled circles) and CHOrH_2Leu^{124}Ala cells (open circles) were exposed to increasing concentrations of cimetidine for 10 mins at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes, pH 7.4. Data represent the mean ± s.e.mean of 4 independent experiments.

[^{125}I]-APT binding sites (83 ± 9% upregulation) (Table 4). Yet, in CHOrH$_2$WT6 and CHOrH$_2$Leu^{124}Ala cells no significant increase in [^{125}I]-APT binding sites was recorded after long-term incubation (24 hrs) with 100 μM cimetidine (Table 4).

Effect of H_2 antagonists on basal cAMP levels and on [^{125}I]-APT binding in CHOrH$_2$WT cells

Besides cimetidine other H_2 antagonists were tested for their ability to induce negative intrinsic activity. As can be seen in Fig. 4, a dose-dependent decrease of basal cAMP levels was observed when CHOrH$_2$WT cells were incubated with increasing concentrations of ranitidine (maximum decrease 88 ± 3%, EC_{50} value: 0.1 ± 0.08 μM, mean ± s.e.mean, n = 4) and famotidine (famotidine-induced maximum decrease 89 ± 3%, EC_{50} value: 42 ± 8 nM, mean ± s.e.mean, n = 4). Remarkably, burimamide did not display negative intrinsic activity (Figs. 4, 5). As can be seen in Fig. 5, 100 μM burimamide was able to inhibit the histamine (30 nM)-induced cAMP production in CHOrH$_2$WT cells significantly. In addition, 100 μM burimamide was able to significantly inhibit the cimetidine (1 μM)-induced reduction of basal levels of cAMP (Fig. 5).

Accordingly, long-term incubation (24 hrs) of CHOrH$_2$WT cells with 10 μM ranitidine and 10 μM famotidine was shown to induce an increase in [^{125}I]-APT binding sites (ranitidine: 55 ± 3% upregulation, mean ± s.e.mean, n = 6, $p < 0.05$, famotidine: 59 ± 7% upregulation, mean ± s.e.mean, n = 7, $p < 0.05$), whereas burimamide (1 mM) did not induce a significant change in H_2 receptor density (burimamide: -4 ± 1% upregulation, mean ± s.e.mean, n = 7).

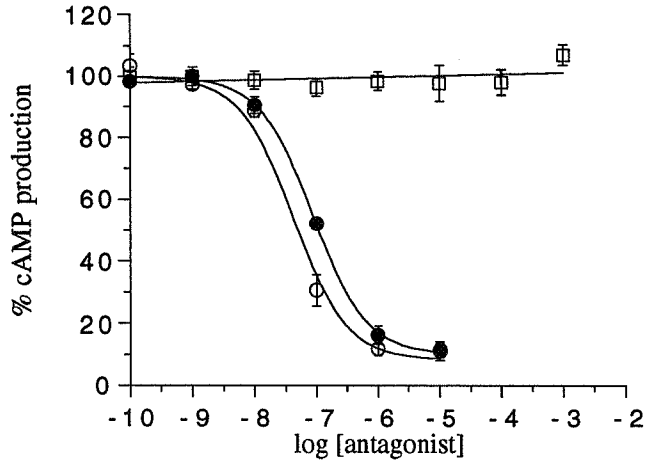


Fig.4. Effect of H_2 antagonists on basal levels of cAMP in CHOrH$_2$WT cells. CHOrH$_2$WT cells were exposed to increasing concentrations of ranitidine (filled circles), famotidine (open circles) or burimamide (open squares) for 10 mins at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes, pH 7.4. Data represent the mean ± s.e.mean of 4 independent experiments.

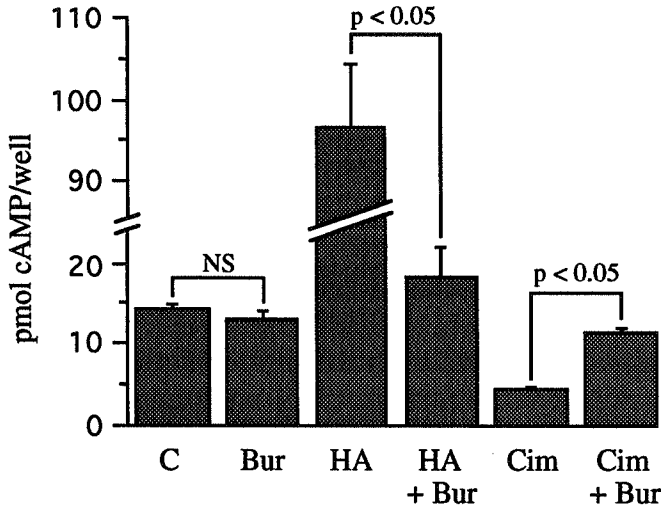


Fig. 5. Effects of burimamide, histamine and cimetidine on basal levels of cAMP in CHOrH_2WT cells. CHOrH_2WT cells were exposed to either 100 μM burimamide (Bur), 30 nM histamine (HA) or 1 μM cimetidine (Cim) or a combination of two of each for 10 mins at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes, pH 7.4. Data represent the mean ± s.e.mean of 3 independent experiments.

Discussion

In the present study we have shown that prolonged exposure (24 hrs) of cells expressing wild-type H_2 receptors to some H_2 antagonists affected the H_2 receptor density. Long-term incubation (24 hrs) of CHOrH_2WT cells with several H_2 antagonists resulted in a marked increase of [^{125}I]-APT binding, referred to as H_2 receptor upregulation. Prolonged treatment of CHOrH_2WT cells with cimetidine resulted in time-dependent ($t_{1/2}$ = 3.5 hrs, concentration of 100 μM cimetidine) and dose-dependent (EC_{50} = 6.0 μM at 24 hrs incubation) H_2 receptor upregulation. Moreover, the lack of H_2 receptor upregulation induced by VUF 8299, a structural analogue of cimetidine which is devoid of H_2 receptor activity, indicates that this process is confined to an H_2 antagonistic effect. In addition, cimetidine had no effect on CHO cells expressing H_1 receptors using the same expression vector. Furthermore, no change in H_2 receptor density was observed after long-term treatment of CHOrH_2WT cells with the H_1 antagonist triprolidine or the H_3 antagonist thioperamide.

The observed effect of these H_2 receptor antagonists might be ascribed to an inhibition of the agonist-induced receptor activity. The antagonist-induced upregulation of the m2- and m3-muscarinic receptors in cultured granule cells (Fukamauchi *et al.*, 1993) was suggested to be explained by a tonic activation of these receptors due to spontaneous synaptic activity between interacting neurons. Nevertheless, there are other reports describing antagonist-induced

upregulation of GPCRs, which cannot be explained by an endogenous agonist-induced receptor activity (Motomura *et al.*, 1990, Yoburn *et al.*, 1994). As for the H_2 receptor expressed in CHO cells, it is unlikely that histamine, leading to a histamine-induced H_2 receptor activity, is present in the medium. Cells were grown in medium with dialyzed foetal calf serum and 24 hrs incubations were performed in serum-free DMEM. Moreover, incubation of CHOrH_2WT cells with 1 nM of histamine was shown to result in a significant elevation of cAMP levels (basal cAMP level: 2.5 ± 0.6 pmol/well, histamine (1 nM)-induced cAMP formation: 9 ± 4 pmol/well), indicating that concentrations of histamine in the medium seem to be lower than 1 nM. In addition, no change in basal cAMP levels were observed when CHOrH_2WT cells were extensively washed and incubated in HBSS medium. The observed H_2 antagonist-induced H_2 receptor upregulation can therefore not be caused by an antagonism of histamine-induced H_2 receptor stimulation.

Interestingly, as already stated in the Introduction, several laboratories have provided evidence for the existence of precoupling of some wild-type GPCRs to G-proteins, thereby exhibiting spontaneous, agonist-independent activity (Barker *et al.*, 1994, Chidiac *et al.*, 1994, Leeb-Lundberg *et al.*, 1994, Tian *et al.*, 1994). Antagonists, shown to favour binding to the inactive state of the receptor, provoke the dissociation of these spontaneously occurring receptor-G-protein complexes, thereby inhibiting basal receptor activity (Chidiac *et al.*, 1994, Samama *et al.*, 1994). These antagonists are referred to as inverse agonists. Antagonists which do not distinguish between the active and inactive state of the receptor do not affect basal receptor activity and are referred to as neutral antagonists. In view of these findings we studied whether the CHOrH_2WT cells exhibited basal, agonist-independent H_2 receptor activity as a consequence of H_2 receptor expression. Moreover, we investigated whether the used H_2 antagonists acted as inverse agonists, thereby causing H_2 receptor upregulation.

As shown in this study, both the basal levels of cAMP and the forskolin-induced cAMP formation increased upon elevation of H_2 receptor density. These observations demonstrate that the wild-type H_2 receptor displays properties resembling those of precoupled GPCRs (Adie and Milligan, 1994a,b, 1994, Barker *et al.*, 1994, Chidiac *et al.*, 1994, Eason *et al.*, 1992, Kim *et al.*, 1995, Leeb-Lundberg *et al.*, 1994, Samama *et al.*, 1993, Tian *et al.*, 1994) and constitutively active GPCR mutants (Cotecchia *et al.*, 1990, Kjelsberg *et al.*, 1992, Ren *et al.*, 1993, Samama *et al.*, 1994). It should be noted, however, that in the published cellular systems, expressing GPCRs coupled to G_s proteins, increased basal and forskolin-induced adenylyl cyclase activity were only recorded at receptor densities exceeding expression levels of 1 pmol/mg protein (Adie and Milligan, 1994a,b, 1994, Kim *et al.*, 1995, Samama *et al.*, 1993). As such, we show that for the H_2 receptor already at a density of 286 fmol/mg protein a significant increase of basal and forskolin-induced cAMP formation is noticed. It is therefore likely that agonist-independent H_2 receptor activity may also be observed at physiological H_2 receptor densities (200-300 fmol/mg protein). Evidence of H_2

receptor precoupling was further supported by experiments using the $H_2Leu^{124}Ala$ receptor mutant. Previously, we observed that this mutation resulted in a loss of agonist high-affinity binding sites and a concomitant loss of histamine potency to induce the production of cAMP (Chapter 5). CHO cells expressing this H_2 receptor mutant ($CHOrH_2Leu^{124}Ala$ cells) displayed reduced basal levels and forskolin-induced formation of cAMP, even though expression levels were comparable to those in $CHOrH_2WT$ cells.

Interestingly, the agonist-independent H_2 receptor activity in $CHOrH_2WT$ and $CHOrH_2WT9$ cells was accompanied by the occurrence of inverse agonism exerted by cimetidine. Its structural analogue VUF 8299, which did not show H_2 antagonism or H_2 receptor upregulation, did not display any negative intrinsic activity in $CHOrH_2WT$ cells. Moreover, in $CHOrH_2Leu^{124}Ala$ and $CHOrH_2WT6$ cells, both displaying reduced basal levels and forskolin-induced formation of cAMP compared to $CHOrH_2WT$ and $CHOrH_2WT9$ cells, cimetidine did not show any negative intrinsic activity. Based on these findings we conclude that binding of cimetidine provokes dissociation of the H_2 receptor-G-protein in those cell lines displaying increased basal adenylyl cyclase activity. As observed earlier (Chapter 5), H_2 receptor density is readily regulated upon receptor stimulation. We therefore hypothesize that the observed inverse agonism displayed by cimetidine could be a mechanistic basis for the observed H_2 receptor upregulation. This hypothesis was supported by the absence of cimetidine-induced H_2 receptor upregulation in $CHOrH_2WT6$ and $CHOrH_2Leu^{124}Ala$ cells. Moreover, not all H_2 antagonists appeared to act as inverse agonists. Interestingly, burimamide was shown to act as a neutral antagonist, as no major changes in basal cAMP levels were observed or in the H_2 receptor density upon 24 hrs incubation. According to the characteristics of a neutral antagonist, burimamide blocked the histamine-induced formation of cAMP and also the cimetidine-induced decrease of basal levels of cAMP. Moreover, the lack of upregulation by burimamide furthermore excludes the possibility that the H_2 receptor upregulation is caused by a competitive antagonism of histamine-induced H_2 receptor stimulation. Famotidine and ranitidine displayed like cimetidine negative intrinsic activity with EC_{50} values that correlate with their respective K_i values (Chapter 5, Traiffort *et al.*, 1992) (ranitidine: EC_{50} value 0.11 μM, K_i value 0.23 μM; famotidine: EC_{50} value 42 nM, K_i value 12 nM; cimetidine: EC_{50} value 1.2 μM, K_i value 0.80 μM).

The mechanism underlying the inverse agonist-induced H_2 receptor upregulation may be attributed to a rapid transient increase of H_2 mRNA levels observed after 0.5 hr cimetidine-treatment. Experiments using protein synthesis inhibitors should give more insight in the mechanism underlying H_2 receptor upregulation.

H_2 antagonists are widely used for the treatment of gastric ulcers (Bertaccini and Coruzzi, 1992). They are considered as one of the safest classes of compounds that has ever been introduced (Deakin and Williams, 1992). However, as already stated in the Introduction, there are some reports that describe observations after long-term H_2 antagonist treatment *in vivo*, which may be explained by the findings obtained in this study (Bertaccini and Coruzzi, 1992,

Coruzzi and Bertaccini, 1989, Martinez-Mir *et al.*, 1993, Merki and Wilder-Smith, 1994, Nwokolo *et al.*, 1991). The inverse agonism displayed by ranitidine, which was paralleled by an increase of H_2 receptors in CHOrH_2WT cells, may explain the increase of parietal cell sensitivity towards dimaprit observed after chronic ranitidine treatment (Coruzzi and Bertaccini, 1989). Moreover, the inverse agonism may also explain the loss of antisecretory effect after prolonged infusions of ranitidine (Merki and Wilder-Smith, 1994) and the observed increase of intragastric hyperacidicity after abrupt ranitidine or cimetidine withdrawal (Nwokolo *et al.*, 1991). Furthermore, neuroleptics, shown to induce an increase of H_2 receptors in brain tissue of schizophrenic patients (Martinez-Mir *et al.*, 1993) and shown to display considerable affinity for H_2 receptors (Leurs *et al.*, 1994, Traiffort *et al.*, 1992), may actually behave as inverse H_2 agonists. Preliminary results in our laboratory have shown that some neuroleptics indeed behave as inverse agonists in CHOrH_2WT cells (M.J. Smit, unpublished observations), possibly explaining the observed increase in H_2 receptor density.

Precoupling of H_2 receptors, revealing inverse agonism of H_2 antagonists, is not necessarily dependent on H_2 receptor expression levels, but may also be induced by G-protein overexpression (Burstein *et al.*, 1995). Recently, overexpression of G_q was shown to induce constitutive activity of muscarine m3 receptors, which was blocked by muscarinic antagonists (Burstein *et al.*, 1995). Moreover, H_2 receptor precoupling properties may be tissue-specific and differ among species and even individuals. The latter may explain why H_2 antagonists usually do not display signs of inverse agonism in the human heart. However, some groups have described negative inotropic effects by H_2 antagonists in some model systems (e.g. guinea-pig atria) and some even in patients, for which so far no explanation could be given (Bertaccini *et al.*, 1993, Hinrichsen and Kirch, 1994). The occurrence of precoupling of H_2 receptors could be a possible explanation.

Taken together, this study shows that at physiological H_2 receptor densities H_2 receptors are precoupled and that several H_2 antagonists, previously thought to act as competitive antagonists, may actually function as inverse agonists. The inverse agonism of H_2 antagonists appears to be a mechanistic basis for the observed H_2 antagonist-induced H_2 receptor upregulation in CHO cells. Moreover, it may also explain the previously reported observations after long-term H_2 antagonist treatment of gastric ulcers such as increased sensitization of the H_2 receptor, increased intragastric hyperacidicity after ranitidine and cimetidine withdrawal and loss of antisecretory effect after ranitidine infusions (Bertaccini and Coruzzi, 1992, Coruzzi and Bertaccini, 1989, Merki and Wilder-Smith, 1994, Nwokolo *et al.*, 1991). It is clear that the therapeutically important class of H_2 antagonists needs reclassification, which may have important clinical consequences. Neutral H_2 antagonists would be favoured in the treatment of gastric ulcers, since increased sensitivity and intragastric hyperacidicity upon withdrawal or loss of H_2 antagonistic effect can then perhaps be avoided.

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Chapter 10

Summary and final considerations

Regulation of receptor function appears to be an integral part of transmembrane signalling of many G-protein coupled receptors (GPCRs) and is important for proper cellular communication (Chapter 1, section 1.3). Moreover, receptor regulation may also have consequences for the development of effective drug therapy. One distinguishes three mechanistically and temporarily distinct regulatory processes. The first involves a rapid (secs to mins) uncoupling of GPCRs from their effector system (short-term desensitization), the second is characterized by the translocation of these receptors away from the cellular surface within minutes (internalization) and the third as a reduction of receptor binding sites occurring after hours of agonist exposure (downregulation or long-term desensitization). These processes are thought to be associated with post-translational modification of the receptor by phosphorylation by kinases, such as protein kinase A (PKA), protein kinase C (PKC) and specific GPCR kinases (GRKs).

Regulation of histamine H_1 and H_2 receptors, both belonging to the class of GPCRs, has not only been observed *in vitro* but also *in vivo* under pathophysiological conditions (e.g. asthmatic attack or allergic reaction). In addition, *in vivo* modulation of the H_1 and H_2 receptor function has also been observed after antagonist treatment. In view of these findings and the widespread use of H_1 antagonists (treatment of allergies) and H_2 antagonists (treatment of gastric ulcers) and the proposed use of H_2 agonists (treatment of congestive heart failure), detailed knowledge of the regulation of the histamine receptors is indispensable. Isolated cell lines and transfected cell lines appear to be excellent model systems for the investigation of (histamine) receptor regulation. Both contain a homogeneous population of either H_1 or H_2 receptors and allow the study of the pharmacological and biochemical properties of these receptors. Additionally, the use of transfected cell lines allows the expression of receptor mutants, which can provide mechanistic insights in phenomena like receptor regulation.

Short-term desensitization of the histamine H_1 and H_2 receptors

In our initial studies we used isolated cell lines of human origin expressing endogenous H_1 or H_2 receptors as model systems to examine the phenomenon of short-term receptor desensitization. For the histamine H_1 and H_2 receptors, the HeLa cell line, a uterine carcinoma cell line, and U937 cell line, a monocytic cell line, were chosen respectively. The H_1 receptors in the HeLa cell line appeared to be readily desensitized upon short-term exposure to histamine as shown by a reduction of the release and influx component of the histamine-induced Ca^{2+} response (Chapter 2). The short-term desensitization of the H_1 receptor was associated with a selective reduction of the release component, mediated by a PKC-independent pathway, and a

non-selective inhibition of the Ca^{2+} influx, activated by a PKC-dependent pathway. As such, short-term desensitization of the histamine H_1 receptor in HeLa cells evolves from two different processes: a PKC-dependent and a PKC-independent pathway (Chapter 2). Similarly, the histamine H_2 receptor-mediated cAMP response in U937 cells was found to be time- and dose-dependently impaired when cells were pretreated with histamine (Chapter 4). The observed H_2 receptor desensitization appeared to be homologous, as β-adrenoceptor-mediated responses remained unaffected.

During the course of these investigations the genes encoding the H_1 and H_2 receptor were cloned. Their deduced amino acid sequences revealed the existence of the commonly reported features of GPCRs: seven putative hydrophobic transmembrane domains, separated by hydrophilic intra- and extracellular loops. Interestingly, the third intracellular loop and cytoplasmatic tail of both receptors were found to contain several serine and threonine residues serving as potential phosphorylation sites for GRKs, as shown for other GPCRs. As such, the observed homologous desensitization of the H_1 and H_2 receptor may be caused by the phosphorylation of these potential phosphorylation sites by GRKs. The development of cell lines expressing epitope-tagged histamine H_1 or H_2 receptors (Chapter 8), thereby allowing direct phosphorylation studies by means of immunoprecipitation, and coexpression of GRKs will provide more insight in the phenomenon of short-term, agonist-induced H_1 and H_2 receptor desensitization. Moreover, the use of histamine H_1 and H_2 receptor mutants, with deletions in the third intracellular loop or truncation of the C-terminal tail (Chapter 6) might give more insight in the structural domains involved in this phenomenon.

Downregulation of the histamine H_1 and H_2 receptors

Initial studies in our laboratory (1991-1992) have shown that the HeLa cell line does not express sufficient levels of H_1 receptor nor does the U937 cell line express sufficient H_2 receptors (~ 60 and ~ 10 fmol/mg protein respectively) to determine the effect of long-term histamine exposure on H_1 or H_2 receptor expression. With the cloning of the genes/cDNAs encoding the H_1 and H_2 receptor proteins, cellular systems expressing sufficient amounts of H_1 or H_2 receptors are currently available. As for the H_1 receptor, CHO cells were transfected with the recently cloned human H_1 receptor, obtaining a CHO cell line expressing human H_1 receptors (CHOhumH_1 cells) at a density of 861 fmol/mg protein (Chapter 3). The human H_1 receptor gene expressed in CHO cells was found to encode a classical H_1 receptor with properties similar to that of the H_1 receptor found in guinea-pig cerebellum and the endogenously expressed human H_1 receptor in 1321N1 astrocytoma cells (Chapter 3). This receptor was also found to be susceptible to short-term receptor desensitization as shown by a decrease of the histamine-induced Ca^{2+} response after histamine exposure. Long-term exposure of the CHOhumH_1 cells to histamine resulted in a dose- and time-dependent downregulation of the human H_1 receptor. As a result of long-term histamine exposure, both histamine- and ATP-induced Ca^{2+} responses were affected, indicating modulations at a level

distant from the receptor too. PKC does not seem to play a role in either the histamine-induced H_1 receptor desensitization or downregulation in CHOhumH_1 cells, which may be explained by a differential expression of PKC isoenzymes in this cell line compared to other cellular systems. Currently, no detailed information is available on the structural domains involved in H_1 receptor downregulation. However, the generation of H_1 receptor mutants analogous to those described for the H_2 receptor (Chapter 5, 6 and 8) will provide more insight in this phenomenon.

Likewise, long-term histamine-exposure of CHO cells expressing the rat histamine H_2 receptor (CHOrH_2 cells, 975 fmol/mg protein) resulted in a dose-and time-dependent reduction of the number of H_2 receptors. H_2 receptor downregulation was also observed upon long-term incubation of CHOrH_2 cells with agents elevating the intracellular cAMP concentration, such as forskolin and cholera toxin. Both histamine and forskolin induced rapid downregulation of H_2 receptor mRNA levels, most likely caused by mRNA destabilization, thereby contributing to the observed decrease of H_2 receptor binding sites. A comparison of the time course and the maximum extent of the histamine- and forskolin-induced H_2 receptor and H_2 mRNA downregulation suggested the involvement of cAMP in the process of H_2 receptor downregulation. However, two H_2 receptor mutants displaying impaired ability to induce cAMP upon histamine exposure (H_2Leu^{124}Ala (Chapter 5) and H_2T341 (Chapter 6)) provided evidence for a cAMP-independent pathway in the process of agonist-induced H_2 receptor downregulation. Both H_2 receptor mutants were shown to be downregulated by histamine, while no or little increase of cAMP was observed. Thus, H_2 receptor downregulation appears to be induced by two distinct pathways, a cAMP-dependent and a cAMP-independent pathway.

Recent studies have shown that besides inducing H_2 receptor downregulation long-term (24 hrs) exposure of CHOrH_2 cells (975 fmol/mg protein) to histamine (100 μM) resulted in a rightward shift of the dose-response curve of histamine-induced cAMP production (Fig. 1). The observed shift cannot be ascribed to decreased adenylyl cyclase activity as forskolin-dose response curves remained unaffected after a long-term histamine exposure (data not shown in this thesis). The shift cannot be explained either by the decrease in H_2 receptor expression, as the EC_{50} value of the histamine-induced cAMP response in CHO cells expressing rat H_2 receptors at a density of approximately 500 fmol/mg protein (50% of density in CHOrH_2 cells) was similar to the value recorded in CHOrH_2 cells (M.J. Smit, unpublished observations). The observed shift might be attributed to a decrease in G_s-protein expression, as was previously reported for other GPCRs upon long-term agonist-exposure. Quantification of G_s-protein levels in histamine-treated cells (24 hrs) should give more insight in the above-mentioned. Thus, histamine-induced H_2 receptor downregulation does not seem to have an immediate effect on H_2 receptor signalling. These experiments, however, suggest modification of e.g. G_s-protein expression upon long-term histamine-exposure, indicating the complexity of

agonist-induced receptor regulation.

These findings do not mean that H_2 receptor downregulation has no physiological meaning. In preliminary studies we observed in CHO cells that the EC_{50} value for the histamine-induced cAMP production is highly correlated with the H_2 receptor density up to 200 fmol/mg protein. (M.J. Smit, unpublished observations). Histamine-induced H_2 receptor downregulation may thus have a marked effect on H_2 receptor signalling in cells expressing low levels of the H_2 receptor protein.

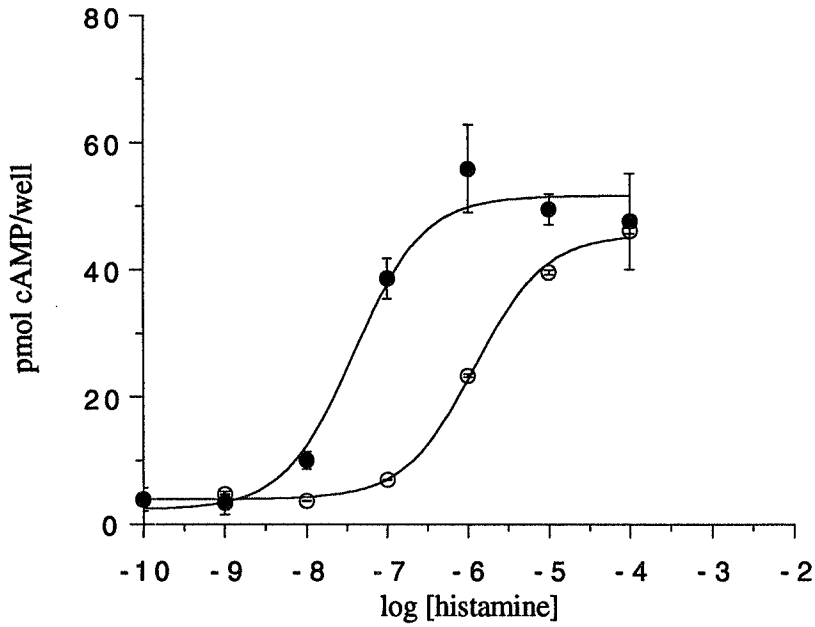

Fig.1. Effect of long-term treatment of $CHOrH_2$ cells with histamine on H_2 receptor signalling. $CHOrH_2$ cells were treated with (open circles) or without (filled circles) 100 μM histamine for 24 hrs in DMEM without foetal calf serum. Thereafter, cells were washed several times and incubated for 1 hr with DMEM supplemented with 25 mM Hepes, pH 7.4. $CHOrH_2$ cells were subsequently incubated with increasing concentrations of histamine for 10 mins at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes, pH 7.4. The levels of cAMP were determined as described in chapter 5.

For several GPCRs the C-terminal tail is considered to be an important structural domain for GPCR regulation. Truncation of the C-terminal tail of the H_2 receptor by 51 amino acids, in contrast to most other GPCRs, resulted in the generation of a mutant H_2 receptor (H_2T307, Chapter 6) which was more susceptible to agonist-induced receptor downregulation, but not to the cAMP-dependent H_2 receptor downregulation. Thus, this region of the C-terminal tail seems to impair agonist-induced H_2 receptor downregulation. Accordingly, the region

encompassing the amino acids 307 to 341 of the H_2 receptor was found to inhibit histamine-induced signalling, suggesting the presence of positive and negative signals in the C-terminal tail of the H_2 receptor for signal transduction and receptor downregulation. Furthermore, these data again indicate that the cAMP-dependent and cAMP-independent pathways of H_2 receptor appear to be mediated by different regulatory processes.

Internalization of the histamine H_2 receptor

Due to a lack of suitable radioligands and anti-H_2 receptor antibodies no studies on H_2 receptor internalization have been reported so far. In order to study the process of H_2 receptor internalization rat and human histamine H_2 receptors were tagged at the N-terminus with the eight amino acid Flag epitope to allow the immunological identification of the receptor peptide with the monoclonal anti-Flag M2 antibody (Chapter 8). Initially, we tried to express the epitope-tagged H_2 receptor in CHO cells. However, no clones expressing the epitope-tagged H_2 receptor were obtained when we used various transfection methods. As such, we stably transfected the epitope-tagged H_2 receptor in human embryonal 293 kidney cells (293rH_2Flag). Importantly, the introduction of the epitope did not affect H_2 agonist or H_2 antagonist binding nor did it affect histamine-induced signalling. Immunofluorescence measurements of 293rH_2Flag cells revealed the presence of anti-Flag-immunoreactivity in the plasma membrane (Chapter 8). Permeabilization of the 293rH_2Flag cells also showed immunofluorescence in the cytoplasm in areas corresponding to the Golgi apparatus (unpublished observations). Using this technique we observed after a one-hour histamine-treatment a complete disappearance of immunoreactivity in the membrane, indicating H_2 receptor internalization. As immunofluorescence of epitope-tagged H_2 receptors was already detected inside the cell it was impossible to identify a further increase of the intracellular immunofluorescence. The receptor internalization was reversible and blocked by the endocytosis inhibitor phenylarsine oxide. Forskolin did not induce H_2 receptor internalization, which indicates that histamine causes H_2 receptor internalization via a cAMP-independent pathway. Internalization of other GPCRs was found to be associated with phosphorylation of serine and threonine domains in the C-terminal tail by kinases other than the second-messenger-dependent kinases. As such, by using an epitope-tagged truncated H_2 receptor mutant (H_2T307, Chapter 6) it would be interesting to investigate whether these serines and threonine residues are involved in the process of H_2 receptor internalization. Moreover, it is as yet not clear whether GPCR internalization requires G-protein coupling. To study the latter, the use of an epitope-tagged uncoupled H_2 receptor mutant (H_2Leu^{124}Ala, Chapter 5) might elucidate whether G-protein coupling is a prerequisite in the process of H_2 receptor internalization.

Antagonist-induced histamine H_2 receptor upregulation

In the chapters 2 to 8 we have shown that the H_1 and H_2 receptors are susceptible both to short-term and long-term desensitization, which results in a decrease of H_1 and H_2 receptor responsiveness and downregulation respectively. In Chapter 9 we demonstrate that also several H_2 antagonists may affect the H_2 receptor function as shown by H_2 antagonist-induced H_2 receptor upregulation. At physiological H_2 receptor densities H_2 receptors were found to be precoupled in CHO cells. Several H_2 antagonists which were previously thought to act as competitive antagonists were shown to inhibit basal H_2 receptor activity, displaying negative intrinsic properties also referred to as inverse agonism. As shown in Chapter 5, H_2 receptors are regulated upon receptor stimulation; inverse agonism of H_2 antagonists may be a mechanistic basis for the observed H_2 receptor upregulation. This hypothesis is supported by both the lack of H_2 receptor upregulation in cells which do not show precoupling ($CHOrH_2Leu^{124}Ala$ and $CHOrH_2WT6$) and the lack of upregulation by neutral H_2 antagonists (e.g. burimamide). Our findings may explain the previously reported observations after long-term H_2 antagonist treatment of gastric ulcers. Several authors reported increased sensitization of the H_2 receptor, increased intragastric hyperacidicity and loss of antisecretory effect after prolonged H_2 receptor blockade.

So far there are no reports describing inverse agonism by H_2 antagonists in other *in vitro* or *in vivo* pharmacological models. However, H_2 receptor precoupling properties may be tissue-specific, differ among species and even individuals. In this respect the level of H_2 receptor expression is important, but also other factors (e.g. G protein levels) might contribute to this phenomenon. It is clear, however, that the therapeutically important class of H_2 antagonists needs a pharmacological reclassification, which may have important clinical consequences. These findings await studies defining the structural components of H_2 antagonists and structural determinants within the H_2 receptor protein responsible for inverse agonism.

While studies on rat histamine H_2 receptor regulation were in progress, also the human H_2 receptor was cloned. Subsequently, this receptor was stably expressed in CHO cells in our laboratory (Chapter 7). Pharmacological studies showed that the cloned human gene encoded a histamine H_2 receptor that is indistinguishable from the H_2 receptor identified in human brain tissue and showed functional coupling to the adenylate cyclase in CHO cells (Chapter 7). Long-term exposure of CHO cells expressing human H_2 receptors to histamine or H_2 antagonists was also found to induce H_2 receptor downregulation or upregulation (M.J. Smit, unpublished observations), validating the use of CHO cells expressing rat H_2 receptors as our model system. Moreover, the use of this model system has emphasized the importance of H_2 receptor mutants (Chapter 5, 6, 8 and 9) in the investigation of mechanisms involved in H_2 receptor signalling and H_2 receptor regulation. However, one should not exclude the investigation of these phenomena in more appropriate models, such as primary cultures or

tissues which more accurately reflect the situation *in vivo*. In order to elucidate the mechanisms underlying histamine receptor function, a combination of both model systems is indispensable.

In conclusion, histamine H_1 and H_2 receptors are susceptible to dynamic receptor regulation. Detailed knowledge of the molecular entities involved in histamine receptor regulation, along with the knowledge regarding both their cellular distribution and their expression levels is required for the development of effective drug therapy.

To be continued!!

Samenvatting en discussie

Signaaltransductie door G-eiwit-gekoppelde receptoren speelt een belangrijke rol bij de communicatie tussen cellen. Voor een effectief verloop van deze communicatie is een goede regulatie van de receptorfunctie essentieel.

Tot de klasse van G-eiwit-gekoppelde receptoren behoren ook de histamine H_1- en H_2-receptor. Regulatie van deze receptoren wordt niet alleen *in vitro,* maar onder pathofysiologische condities (astmatische aanval, allergische reactie) ook *in vivo* waargenomen. Bovendien worden *in vivo* veranderingen gezien in H_1- en H_2-receptorfunctie na behandeling met histamine-antagonisten. Deze antagonisten worden onder meer gebruikt voor de behandeling van allergieën (H_1) en van maagzweren (H_2). Sommige auteurs stellen voor H_2-agonisten toe te dienen aan patienten met hartinsufficiëntie. Omdat medicijnen receptorregulatie kunnen geven, is inzicht in de mechanismen die daaraan ten grondslag liggen onmisbaar voor de ontwikkeling van effectieve therapieën.

We onderscheiden drie verschillende regulatiemechanismen, die onderling verschillen in tijdsverloop en karakter. Het eerste mechanisme noemen we kortdurende desensitisatie. Dit mechanisme wordt gekarakteriseerd door een snelle (seconden tot minuten) ontkoppeling van de G-eiwit-gekoppelde receptoren van hun effectorsysteem. Het tweede mechanisme, internalisatie, uit zich in een translocatie van receptoren aan het celoppervlak naar het cytoplasma. Het derde mechanisme tot slot speelt zich pas na uren af en resulteert in een afname van het aantal receptoren. Dit mechanisme noemen we downregulatie. Kinases, zoals proteïne kinase A (PKA), proteïne kinase C (PKC) en specifieke G-eiwit-gekoppelde receptorkinases blijken bij de verschillende regulatiemechanismen betrokken te zijn.

Geïsoleerde en getransfecteerde celsystemen zijn uitermate geschikt als modelsysteem voor het onderzoek naar de regulatie van histamine H_1- en H_2-receptoren. Beide bezitten een homogene populatie van histamine-receptoren, waardoor farmacologische en biochemische eigenschappen goed kunnen worden bestudeerd. Bovendien kunnen receptormutanten, die essentieel zijn voor de opheldering van regulatiemechanismen, in getransfecteerde celsystemen tot expressie worden gebracht en daarna functioneel worden onderzocht.

Kortdurende desensitisatie van histamine H_1- en H_2-receptoren

Het onderzoek is gestart met geïsoleerde celsytemen van humane afkomst, die endogene H_1- of endogene H_2-receptoren tot expressie brengen. Voor onderzoek naar de H_1-receptor was dit de HeLa-cellijn, een uit de baarmoeder afkomstige carcinome cellijn, en voor de H_2-receptor de U937-cellijn, een monocytachtige. De H_1-receptoren op de HeLa-cellijn blijken na toediening van histamine snel te desensitiseren, hetgeen zich uit in een verminderde Ca^{2+}-respons. Deze desensitisatie is geassocieerd met een selectieve reductie van de 'Ca^{2+}-release' veroorzaakt door een PKC-onafhankelijk mechanisme, en een niet-selectieve vermindering van de 'Ca^{2+}-

influx', veroorzaakt door een PKC-gemedieerd mechanisme (hoofdstuk 2). Desensitisatie van de H_1-receptor wordt dus gemedieerd door twee verschillende routes: een PKC-onafhankelijke en PKC-afhankelijke.

Bij de H_2-receptor treedt een vergelijkbaar proces op: de histamine-geïnduceerde cAMP-respons in U937-cellen vermindert op een tijds- en dosisafhankelijke wijze (hoofdstuk 4). Deze desensitisatie is homoloog: de β-receptor gemedieerde respons blijft gelijk.

Andere onderzoekers kloneerden in de loop van het onderzoek de genen die coderen voor de H_1- en de H_2-receptor. Uit de aminozuurvolgorde van beide receptoreiwitten blijkt dat deze inderdaad tot de klasse van G-eiwit-gekoppelde receptoren behoren. Beide receptoren zijn opgebouwd uit zeven transmembraandomeinen, die door drie intra- en drie extracellulaire loops worden verbonden. Wat opvalt is dat er zowel in de derde intracellulaire loop als in de C-terminale staart van beide receptoren potentiële serine- en threonineresiduen zitten, die wellicht gefosforyleerd kunnen worden door specifieke receptorkinases. Deze fosforyleringen zouden de homologe desensitisatie van de receptoren kunnen verklaren. Het creëren van een cellijn waarin H_1- of H_2-receptoren met een epitoop eraan (hoofdstuk 8) tot expressie worden gebracht, maakt het mogelijk om - met immuunprecipitaties - directe fosforylering van het desbetreffende receptor-eiwit aan te tonen. Coëxpressie van specifieke receptorkinases met deze epitoop-receptoren kan meer inzicht geven in de agonist-geïnduceerde H_1- en H_2-receptordesensitisatie. Daarnaast zal het gebruik van gemuteerde H_1- of H_2-receptoren, bijvoorbeeld met een deletie in de derde intracellulaire loop of zonder cytoplasmatische staart, veel kunnen verhelderen.

Downregulatie van histamine H_1- en H_2-receptoren

De dichtheid van de H_1-receptoren in de HeLa- en de dichtheid van de H_2-receptoren in de U937-cellijnen (respectievelijk ~60 en ~20 mg fmol/mg eiwit) zijn voor onderzoek naar downregulatie niet hoog genoeg. Tijdens het onderzoek publiceerden andere onderzoekers de nucleotide-sequenties die voor deze receptoren coderen. Met deze kennis konden cellijnen met voldoende hoge expressie worden verkregen.

Het gen dat codeert voor de humane H_1-receptor werd tot expressie gebracht in ovaria cellen van de Chinese hamster (CHO-cellen), wat een cellijn met een dichtheid van 861 fmol/mg eiwit opleverde (CHOhumH_1-cellen). De receptoren bleken dezelfde farmacologische eigenschappen te hebben als H_1-receptoren in het cerebellum van de cavia en de humane endogene H_1-receptoren in de 1321N1 astrocytomacellen (hoofdstuk 3). De receptor was verder gevoelig voor desensitisatie, gezien de afname van de Ca^{2+}-respons na toediening van histamine. Langdurige blootstelling aan histamine leidde tot een dosis- en tijdsafhankelijke afname van het aantal receptoren (downregulatie). Tevens was zowel de door histamine als de door ATP geïnduceerde Ca^{2+}-respons verminderd, hetgeen aangeeft dat modulatie ook plaatsvindt op andere niveaus dan die van de receptor. PKC blijkt echter geen rol te spelen, noch in de desensitisatie, noch in de downregulatie van de H_1-receptor in CHOhumH_1-cellen.

Dit kan verklaard worden door een verschil in expressie van PKC-iso-enzymen in CHOhumH_1-cellen en in andere celsystemen. Er is nog geen informatie over de structurele H_1-receptordomeinen die bij de downregulatie betrokken zijn. Mutanten kunnen meer inzicht geven in downregulatie van de H_1-receptor, zoals ook voor de H_2-receptor is gedaan (hoofdstuk 5, 6 en 8).

Op eenzelfde wijze werd in CHO-cellen de H_2-receptor van de rat tot expressie gebracht (CHOrH_2-cellen). Langdurige toediening van histamine leidde tot een dosis- en tijdsafhankelijke downregulatie van H_2-receptoren. Langdurige blootstelling aan stoffen die de intracellulaire cAMP-concentratie verhogen, zoals forskoline en cholera-toxine, leidde eveneens tot een afname van het aantal H_2-receptoren. Verder zorgen histamine én forskoline voor een snelle downregulatie van mRNA, mogelijk veroorzaakt door destabilisatie van het mRNA. Het verloop en de mate van downregulatie lopen voor de door histamine en forskoline geïnduceerde vermindering gelijk, zowel voor de receptoren als voor het mRNA. Dit wijst erop dat cAMP een belangrijke rol speelt bij de downregulatie van H_2-receptoren. Twee mutanten van de H_2-receptor, H_2Leu^{124}Ala (hoofdstuk 5) en H_2T307 (hoofdstuk 6), toonden echter ook een cAMP-onafhankelijke route aan. Bij beide mutanten was de cAMP-produktie na toediening van histamine minder sterk vergeleken met de wild-type receptor. Toch kon bij concentraties die nog geen verhoging van cAMP veroorzaakten wel een downregulatie worden waargenomen. Downregulatie van de H_2-receptor blijkt dus te worden gemedieerd door twee verschillende routes: een cAMP-afhankelijke en een cAMP-onafhankelijke.

Recente studies hebben aangetoond dat bij langdurige blootstelling (24 uur) van CHOrH_2-cellen aan histamine (100 μM) de dosis-respons-curve van de door histamine geïnduceerde cAMP-respons naar rechts verschuift (Fig. 1). Deze verschuiving kan niet verklaard worden door verminderde activiteit van het adenylaatcyclase, omdat de curve van de door forskoline geïnduceerde cAMP-respons gelijk bleef (niet gepubliceerde resultaten). Downregulatie is evenmin een verklaring: de EC_{50}-waarde van de curve van de door histamine geïnduceerde cAMP-respons blijft gelijk in CHO-cellen die slechts 500 fmol H_2-receptoren per mg eiwit tot expressie brengen (50% van het aantal H_2-receptoren in CHOrH_2-cellen; niet gepubliceerde resultaten). Wel zou een verminderde expressie van het G_s-eiwit de verschuiving kunnen verklaren. Dit is ook beschreven voor andere G-eiwit gekoppelde receptoren. Kwantificering van G_s-eiwit-niveaus na behandeling met histamine gedurende 24 uur kan hierin meer inzicht geven.

De door histamine geïnduceerde downregulatie van H_2-receptoren blijkt dus geen direct effect te hebben op de signaaltransductie van de H_2-receptor. Deze experimenten laten zien hoe complex het proces van agonist-geïnduceerde receptor downregulatie is. De downregulatie is toch fysiologisch belangrijk. Preliminaire studies lieten zien dat de EC_{50}-waarde van de histamine-geïnduceerde cAMP dosis-respons-curve afhankelijk is van de dichtheid van de H_2-receptor tot 200 fmol/mg eiwit (niet gepubliceerde resultaten). De door histamine geïnduceerde

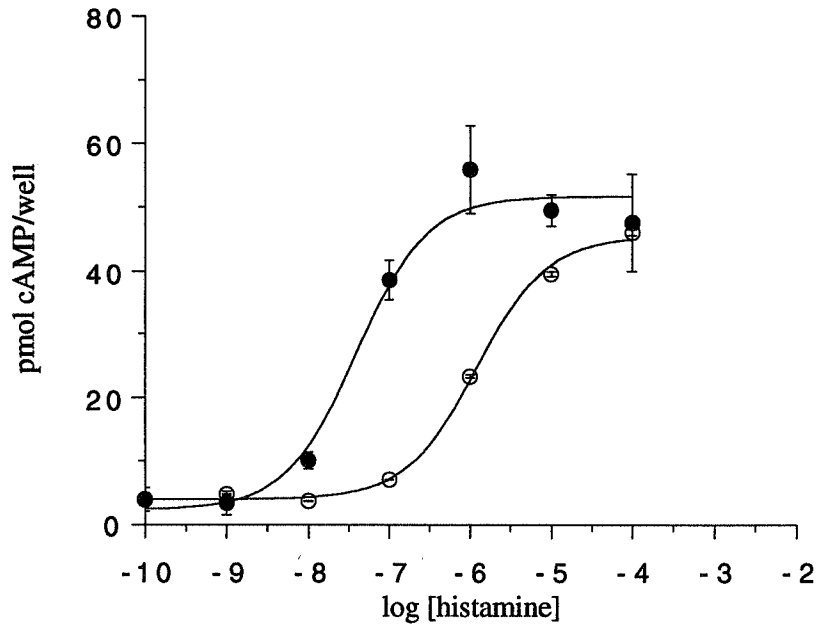

Fig.1. Effect van langdurige histamine behandeling op de H_2-receptor signaaltransductie in $CHOrH_2$-cellen. $CHOrH_2$-cellen werden met (open cirkels) of zonder (gevulde cirkels) 100 µM histamine gedurende 24 uur voorbehandeld in DMEM zonder foetaal kalf serum. Vervolgens, werden cellen vele malen gewassen en geïncubeerd voor 1 uur in DMEM met 25 mM Hepes, pH 7.4. $CHOrH_2$-cellen werden daarna geïncubeerd met oplopende concentraties histamine gedurende 10 minuten bij 37°C in DMEM in aanwezigheid van 300 µM IBMX en 25 mM Hepes, pH 7.4. De cAMP niveaus werden bepaald zoals beschreven in hoofdstuk 5.

downregulatie van H_2-receptoren heeft dus wel degelijk effect op de signaaltransductie in cellen met een lage dichtheid H_2-receptoren.

Voor verschillende G-eiwit-gekoppelde receptoren is gebleken dat de C-terminale staart een belangrijke rol speelt in receptorregulatie. Truncatie van 51 aminozuren van de C-terminale staart van de H_2-receptor genereert een mutant (H_2T307, hoofdstuk 6) die een sterkere agonist-afhankelijke downregulatie geeft, terwijl de cAMP-afhankelijke downregulatie gelijk blijft. Veel andere G-eiwit-gekoppelde receptoren laten in dat geval juist minder downregulatie zien. Dit gedeelte van de C-terminale staart van de H_2-receptor belemmert dus agonist-geïnduceerde downregulatie. Op vergelijkbare wijze is de regio in de C-terminale staart van aminozuur 307 tot aminozuur 341 betrokken bij de remming van de door histamine geïnduceerde signaaltransductie. Dit suggereert dat de C-terminale staart van de H_2-receptor zowel positieve als negatieve signalen bevat voor de signaaltransductie en de downregulatie. Bovendien laat deze H_2-receptormutant zien dat de cAMP-afhankelijke en cAMP-

onafhankelijke H_2-receptor downregulatie via verschillende regulatieprocessen verlopen.

Internalisatie van histamine H_2-receptoren

Omdat geschikte radioliganden en antilichamen ontbreken is de internalisatie van H_2-receptoren tot nu toe niet onderzocht. Om dit fenomeen toch te kunnen bestuderen werd een epitoop van acht aminozuren (Flag) aan de N-terminale zijde van de H_2-receptor vastgemaakt. Daardoor kan de receptor met een monoklonaal anti-Flag-antilichaam worden gedetecteerd (hoofdstuk 8). Het bleek niet mogelijk om de receptor-met-Flag in CHO-cellen tot expressie te brengen. Dit lukte wel in humane embryonale niercellen (293rH_2Flag-cellen). Onderzoek heeft uitgewezen dat de plaatsing van een Flag-eiwit aan de H_2-receptor geen invloed heeft op de bindingseigenschappen van H_2-agonisten en -antagonisten. Ook de histamine-geïnduceerde signaaltransductie verloopt normaal.

Met behulp van immunofluorescentiemetingen werd op het membraan van de 293rH_2Flag-cellen anti-Flag immunofluorescentie waargenomen (hoofdstuk 8). Permeabilisatie van deze cellen gaf ook immunofluorescentie te zien in het cytoplasma, in regio's waar zich het Golgi-apparaat bevindt. Na behandeling van de cellen met histamine (1 uur) was de immunofluorescentie in het membraan geheel verdwenen. Dit duidt op internalisatie van de H_2-receptoren. Omdat ook onder normale omstandigheden (delen van) receptoren in de cel aanwezig zijn, is het helaas niet mogelijk om verhoging van internalisatie met immunofluorescentie aan te tonen.

H_2-receptor-internalisatie is reversibel. Het proces kan geblokkeerd worden door de endocytose-remmer fenylarsine-oxide. Forskoline was niet in staat om de internalisatie te induceren, hetgeen bewijst dat het proces cAMP-onafhankelijk is.

Internalisatie van andere G-eiwit-gekoppelde receptoren gaat gepaard met de fosforylering van serine- en threonineresiduen in de C-terminale staart, door nog niet geïdentificeerde kinases. Het zou daarom interessant zijn een getrunceerde (H_2T307, hoofdstuk 6) H_2-receptor-Flag te gebruiken om te onderzoeken of ook in dit geval de serine- en threonineresiduen in de C-terminale staart bij de internalisatie betrokken zijn. Het is overigens nog niet duidelijk of G-eiwit-koppeling nodig is voor internalisatie. Het gebruik van de ontkoppelde H_2-receptormutant (H_2Leu^{124}Ala, hoofdstuk 5) met daaraan een Flag-epitoop kan hierin helderheid brengen.

Antagonist-geïnduceerde upregulatie van H_2-receptoren

De hoofdstukken 2 t/m 8 laten zien dat H_1- en H_2-receptoren onderhevig zijn aan kort- en langdurige desensitisatie. Dit uit zich in een afname van de receptorrespons en in receptor-downregulatie. Hoofdstuk 9 beschrijft dat ook H_2-*antagonisten* effect hebben op H_2-receptorexpressie. Langdurige blootstelling van CHOrH_2-cellen aan H_2-antagonisten resulteerde in een upregulatie van H_2-receptoren. Bij fysiologische dichtheden van de H_2-receptor in CHO-cellen zijn de receptoren geprekoppeld. Verschillende H_2-antagonisten, die

voorheen als competitief beschouwd werden, blijken nu negatieve intrinsieke activiteit te vertonen: ze zijn in staat om de basale H_2-receptor activiteit te blokkeren. Dit fenomeen wordt ook wel 'invers agonisme' genoemd.

Zoals reeds getoond in hoofdstuk 5 worden H_2-receptoren na stimulatie gedownreguleerd. Invers agonisme van sommige antagonisten kan dus aan de waargenomen upregulatie ten grondslag liggen. Deze hypothese wordt verder ondersteunddoor het feit dat cellen die geen H_2-receptor prekoppeling vertonen (CHOrH_2Leu^{124}Ala en CHOrH_2WT6) geen upregulatie geven. Ook neutrale antagonisten zoals burimamide doen dit niet. Deze resultaten kunnen eerdere publikaties verklaren inzake langdurige behandeling van maagzweren met H_2-antagonisten: sommige onderzoekers zagen in dat geval een verhoogde gevoeligheid van de H_2-receptoren, een verhoogde zuurgraad en een verlies van de remmende werking van H_2-antagonisten. Prekoppeling van receptoren, hetgeen aanleiding geeft tot invers agonisme, is niet noodzakelijk afhankelijk van receptor-expressie, maar kan ook veroorzaakt worden door overexpressie van G-eiwitten. Prekoppeling-eigenschappen kunnen dus specifiek zijn voor het weefsel en per individu verschillen. Het is duidelijk dat de therapeutisch zeer belangrijke H_2-antagonisten opnieuw geclassificeerd dienen te worden, hetgeen belangrijke klinische consequenties kan hebben. Verder onderzoek is nodig naar de structurele componenten van de H_2-antagonisten en de structurele domeinen van het H_2-receptor-eiwit die voor invers antagonisme verantwoordelijk zijn.

In de loop van het onderzoek kloneerden andere auteurs ook het gen dat codeert voor de humane H_2-receptor. Met deze kennis kon de humane H_2-receptor in CHO-cellen tot expressie worden gebracht (CHOhumH_2-cellen) (hoofdstuk 7). Een uitgebreide karakterisering van deze receptor liet zien dat de humane H_2-receptor dezelfde eigenschappen heeft als de H_2-receptor in humane hersenen (hoofdstuk 7). Langdurige blootstelling van CHOhumH_2-cellen aan histamine en H_2-antagonisten resulteerde ook in downregulatie, respectievelijk upregulatie (niet gepubliceerde resultaten). Dit valideert het gebruik van het modelsysteem (CHOrH_2-cellen). Het modelsysteem benadrukt bovendien het belang van het gebruik van H_2-receptormutanten in het onderzoek naar H_2-receptorregulatie (hoofdstuk 5, 6, 8 en 9). Het is zinvol om de modelsystemen die meer de *in vivo* situatie benaderen, zoals primaire culturen of weefsels, in het onderzoek naar regulatieprocessen te betrekken. Een combinatie van *in vitro*- en meer *in vivo*-systemen komt het onderzoek naar de regulatiemechanismen van de histamine H_1- en H_2-receptor sterk ten goede.

Samenvattend: histamine H_1- en H_2-receptoren zijn onderhevig aan een dynamische receptorregulatie. Voor de ontwikkeling van effectieve therapieën is een gedetailleerde kennis van de eiwitten die hierbij betrokken zijn, als ook meer inzicht in hun cellulaire distributie en expressieniveaus noodzakelijk. Wordt vervolgd!!

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Nawoord

Dit proefschrift is het resultaat van een plezierige samenwerking met een groot aantal mensen.

Rob Leurs, jouw proefschrift vormde de basis van dit onderzoek. Je enorme enthousiasme en vakkennis zijn uniek. Onze vriendschappelijke samenwerking was zeer inspirerend. Professor Henk Timmerman bedank ik hartelijk voor zijn nauwe betrokkenheid bij het onderzoek en voor zijn waardevolle suggesties. Professor Aalt Bast bedank ik voor zijn inbreng gedurende de eerste twee jaren van mijn AIO-tijd.

Een speciale vermelding verdienen de studenten, die een belangrijke bijdrage hebben geleverd aan het onderzoek. Steven, Ingrid, Yvonne, Carien, Geerten, Joeri en Astrid, hartelijk dank voor de inzet en het enthousiasme waarmee jullie hebben gewerkt. Margot Beukers dank ik voor de hulp bij het maken van de truncatie-mutanten en Edwin Roovers voor de mRNA-bepalingen die hij heeft gedaan.

Ook ben ik dank verschuldigd aan professor De Laat, Margreet Bloemers en Leon Tertoolen van het Hubrecht Laboratorium in Utrecht. Onze prettige samenwerking heeft geleid tot hoofdstuk 2 van dit proefschrift. Dankzij de samenwerking met Dr. Jan van Minnen en Dr. Misha Pounine van de afdeling Organismale Diergeneeskunde van de faculteit Biologie van de Vrije Universiteit was het mogelijk door middel van immunofluorescentie-microscopie de histamine H_2-receptor te visualiseren (zie omslag van dit proefschrift).

I thank Dr. Fukui for giving me the opportunity to work at the Department of Pharmacology at the University of Osaka, Japan. I am also grateful to Dr. Ito and Dr. Sakamoto for giving me the possibility to do research at the Osaka Bioscience Institute, Japan. Furthermore, I thank Dr. Lohse for my short stay at the Laboratory of Molecular Biology at the Max Planck Institute in München.

Professor Hill, I am honoured that you were the referee for this thesis. Thank you for your useful comments. Professor Velthorst, bedankt voor de aandacht die U als "derde lezer" aan mijn proefschrift heeft geschonken. Ellen Mooijman ben ik dank verschuldigd voor het zorgvuldig nakijken van de veschillende hoofdstukken van dit proefschrift.

Verder bedank ik de (oud-)collega's van de vakgroep Farmacochemie voor de prettige sfeer en de gezelligheid, zowel tijdens als na het werk. Ik wens alle jaargenoten uit de 1986-lichting die met hun promotie-onderzoek bezig zijn daarbij veel succes. Familie en vrienden, bedankt voor jullie belangstelling.

Tot slot, "mijn" Rob, reuze bedankt voor alle steun. Op naar de volgende uitdaging!

Martine Smit